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13997 P

Carrot Diet and Susceptibility to Acute "Anoxia."*

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Campbell[1,2] fed carrots to a group of rats and observed that the diet protected the animals from the effects of acute anoxia. By determining what ingested substances hasten or retard the death of animals exposed to a lethal degree of oxygen deficiency, Campbell believed that he could not only gain insight into the mechanisms involved, but could also devise a diet beneficial for patients suffering from various forms of anoxia.

There is increasing doubt as to the validity of obtaining data under one condition of anoxia and exposure to altitude and conjecturing therefrom as to what would obtain in more or less severe degrees of oxygen want or exposure to other altitudes. Nevertheless the clear cut results of acute studies are valuable in suggesting possibilities to be verified in chronic and less severe states.

The current interest in anoxia prompted us to repeat Campbell's apparently significant observations, especially since he used only relatively small groups of animals.

Experimental. Two hundred and fourteen male rats (194 albino and 20 hooded) were divided into odd and even groups. The mean body weight of the former was 171.3 g and the latter, 173.1. They will be designated as Groups A and B, respectively.

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* This study has been aided by a grant from the Clara A. Abbott Fund of Northwestern University.

[1] Campbell, J. A., *Quart. J. Exp. Physiol.*, 1938, **28**, 231.

[2] Campbell, J. A., *Quart. J. Exp. Physiol.*, 1939, **29**, 259.

The animals of Group A were housed in wire-bottomed cages and fed nothing but fresh carrots and tap water *ad libitum* for 10 days. The animals of Group B had a standard diet of Wayne's Kennel Krums, ground corn, and tap water *ad libitum* to which was added whole milk on the first and fifth experimental days and bread and lettuce on the third and eighth days.

On the eleventh day the animals were reweighed. The mean weight of Group A was 150.6 and that of Group B was 185.8.

The animals were placed in a decompression chamber which was illuminated to permit observation of their behavior. A detailed description of the tank is published elsewhere.[3]

The pressure was reduced to 225.6 mm Hg. (simulated altitude of 30,000 ft.) in 15 min and maintained at that level for 2 hr; after which it was returned to ground level in 45 min.

The temperature in the tank was as follows: 0-0.5 hr, 78°F; 0.5-1 hr, 80°F; 1-1.5 hr, 84°F; 1.5-2.0 hr, 86°F.

Results. Of the 107 carrot-fed rats 85 (79%) survived the exposure to high altitude whereas only 23 of the 107 control animals (21%) survived.

This striking difference in the survival of the treated and untreated groups confirms the result reported by Campbell. Our technic was not exactly that used by Campbell but the differences were obviously unimportant.

Experiments are now in progress which are designed to clarify the factors responsible for the protective effect of the carrot diet.

Summary. One hundred seven rats fed nothing but carrots for 10 days and 107 rats fed a stock diet were subjected to a simulated altitude of 30,000 ft for 2 hr. Eighty percent of the carrot-fed animals survived and only 20% of the controls survived. The animals on the carrot diet lost 1% of their body weight per day.

A diet of nothing but fresh carrots fed to rats for 10 days for some unknown reason affords marked protection against the lethal effects of exposure to a simulated altitude (decompression chamber) of 30,000 ft for 2 hr. This confirms the observations of J. A. Campbell.[1]

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[3] Andrews, A. H., Jr., Roth, L. W., and Ivy, A. C., *Quart. Bull. Northwestern University Medical School*, 1941, **15**, 46.

13998 P

Cardiovascular Effect of Trichlorethylene.

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The neural depressing effect of trichlorethylene[1] can explain the alleviation of pain observed clinically, yet its value in the prevention and treatment of angina pectoris[2] may be also due to a cardiovascular effect. With this possibility in mind, a study of the effect of trichlorethylene on the cardiovascular system was undertaken.

Two reports dealing with the cardiovascular effect of trichlorethylene are available. Both are from the same group of workers.[3,4]

Lever experiments with the frog's heart were first carried out. Both the atrial and ventricular beat were recorded. The results from 6 experiments were alike. When 1 cc of the drug was dropped on the heart, the rate was slowed, and notching, observed in the control record, disappeared. When the heart is washed with physiologic saline the effects of trichlorethylene are removed. If more than 1 cc of the drug is used, the heart continues to slow and stops in about 5 min. Washing with physiologic saline does not then revive the heart.

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[1] Rubenstein, H. S., Painter, E., and Harne, O. G., *J. Lab. and Clin. Med.*, 1939, **24**, 1238.

[2] Love, W. A., *Ann. Int. Med.*, 1937, **10**, 1187.

[3] Krantz, J. C., Carr, C. J., and Harne, W. J., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 334.

[4] Krantz, J. C., Carr, C. J., Musser, R., and Harne, W. G., *J. Pharm. and Exp. Therap.*, 1935, **54**, 327.

Since the electrocardiogram is a better record of the condition of the myocardium and conducting mechanism of the heart, it was decided to use this method next. Rabbits (4) were used for these experiments, and trichlorethylene was administered by inhalation. When the control records were made the rabbits were in a basal-like state,[5] and the drug was administered while they were still in this state. One rabbit was anesthetized every day for a week to study the effect of repeated anesthetizations. In the rabbits not previously treated with trichlorethylene, the drug produced arrhythmias and marked slowing. In the rabbit anesthetized each day for a week, T wave changes were noted on the electrocardiogram taken at the end of the period in addition to those mentioned above.

Experiments were also carried out to determine the effect of trichlorethylene on human and frog capillaries. These were visualized with a capillary microscope and measured with a micrometer. The capillaries of the parietal peritoneum were visualized in the case of the frogs, whereas those of the nail bed of the ring finger of the left hand were used in the case of the human experiments. Capillary loops were observed in all instances in order to study both the arterial and venous divisions. Trichlorethylene was administered by inhalation. Tests were carried out on 6 different human subjects and on 6 different frogs. The results were alike. Constriction of the arterial side of the capillary loops occurred. The degree of constriction was approximately 50%.

All of the aforementioned experiments are being extended and further work is being done.

It was concluded that: (a) trichlorethylene causes vasoconstriction of the arterial division of capillary loops both in humans and frogs; (b) it does not produce permanent injury to the heart unless it is given in large quantities (frog) or repeatedly (rabbit).

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[5] Werch, S. C., *J. Lab. Clin. Med.*, 1941, **26**, 725.

13999 P

Trypanocidal Activity and Arsenic Content of Rat Blood Following Intravenous Administration of Mapharsen.

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Fractional dose therapy of rat trypanosomiasis has shown the trypanocidal action of mapharsen *in vivo* to be of relatively short duration.[1] The brief trypanocidal activity of rabbit serum after administration of the related trivalent arsenical, reduced tryparsamide, is also indicative of this fact.[2] On the contrary, arsenic has been found in the blood and tissues of rats for relatively long periods after mapharsen injection.[3] This study was concerned with an investigation of the apparent lack of parallelism between these two factors.

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* Accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Minnesota. Assistance in the preparation of these materials was furnished by the Personnel of the Work Projects Administration, Official Project No. 165-1-71-440, Subproject No. 347.

[1] Swinyard, A. E., Hirschfelder, A. D., and Wright, H. N., *J. Pharm. Exp. Therap.*, 1942, **75**, 367.

[2] Murgatroyd, F., Russel, H., and Yorke, W., *Ann. Trop. Med. Parasit.*, 1934, **28**, 227.

The procedure consisted in determining the minimum trypanocidal concentration (M.T.C.) of the blood arsenic of rats at various intervals after intravenous injection of the maximum tolerated dose (12.5 mg/kg) of mapharsen and comparing this with the M.T.C. of the uninjected drug. The blood samples were taken at ¼, ½, 1, 4, 8, 12, 18, 24, 36, 48 or 240 hr after injection, heparin being used as an anticoagulant. The arsenic content was determined by the A.O.A.C. Gutzeit method,[4] and was expressed as μg (γ) of mapharsen per cc of blood. The M.T.C. was determined by a modification of the method of Murgatroyd *et al.*,[2] and Hawking *et al.*,[5] sterile technic being used throughout. A series of dilutions of the blood was made, on the basis of arsenic content, with a medium consisting of 1 part of heparinized rat blood and 2 parts of Locke's solution (0.2% glucose). The tubes were then inoculated with a dilute suspension of *Trypanosoma equiperdum* from infected rats and incubated for 18 hr at 37°C. After incubation, cultures which showed no motile organisms in microscopic cover-glass preparations were injected intraperitoneally into young rats, which were observed for septicemia and death for 30 days. As the study was extended, mice were substituted for rats, and found satisfactory in this infectivity test. The minimum concentration of arsenic rendering cultures non-infective was taken as the M.T.C. *(in vivo)*. The M.T.C. of uninjected mapharsen *(in vitro)* was determined simultaneously in each case, using the same diluting medium and the same inoculum of parasites. The mean M.T.C.'s *in vivo* and *in vitro* for any interval were considered different only if they exceeded a significant value (±1:2.018 million) calculated statistically from results on duplicate determinations on mapharsen made on 10 occasions.

The trypanocidal activity of the blood, exceedingly high at first, decreased steadily and disappeared after 24 to 36 hr. The trypanocidal period could be divided into 3 phases:

1. A period of extremely high activity for ½ to 1 hr during which the activity of the blood arsenic did not differ significantly from that of the drug itself, the decrease in activity being due entirely to a decreasing arsenic content. At ½ hr the arsenic content of the blood was 24.45γ per cc. (1:35,419) or 13.8% of the theoretical content immediately after injection. The M.T.C. *in vivo* was 1:11.1 million (m), the M.T.C. *in vitro* being 1:12.2 m.

2. A period of intermediate activity, to about 8 hr, during which the decreasing activity was due both to a decrease in the activity of the arsenic present and to a further decrease in the blood arsenic level. At 8 hr the blood arsenic level was 9.13γ per cc (1:109,509). The M.T.C. *in vivo* was 1:0.34 m, the *in vitro* value being 1:7.5 m.

3. A period of low activity during which the trypanocidal activity of the blood continued to decrease in spite of an increase in the arsenic content. At 12 hr the arsenic level was 11.34γ per cc (1:88,086), the M.T.C. *in vivo* being 1:0.12 m as compared with an *in vitro* value of 1:12.2 m. At 24 hr these values were 19.48, 1:0.055 m, and 1:10.9 m respectively. At 36 and 48 hr the blood was non-trypanocidal in spite of a substantial arsenic content. Arsenic was still present, but inactive, at 240 hr. Wright[3] has shown that the rise in blood arsenic at later intervals is due to a return of arsenic from the tissues. Since this returning arsenic is non-trypanocidal and since the incubation of mapharsen with blood produces a much slower decrease in activity than that occurring *in vivo*, it appears that the tissues play a major role in inactivation of the drug.

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[3] Wright, H. N., unpublished data.

[4] *Methods of Analysis, Association of Official Agricultural Chemists*, 4th Ed., 1935.

[5] Hawking, F., Hennelly, T. J., and Quastel, J. H., *J. Pharm. Exp. Therap.*, 1937, **59**, 157; 1938, **64**, 147.

14000

Thiamin Excretion Tests in Children with Paralytic Poliomyelitis.

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A most important question concerning the natural history of human poliomyelitis is: Why do most individuals infected with poliomyelitis virus escape paralysis, the contact with virus being expressed in them by the non-paralytic, abortive, or inapparent forms of the disease? One answer frequently given is that an immunity has been achieved as the result of previous repeated exposure to subinfective doses of virus, but there is no evidence that subinfective doses *(i.e.,* those which fail to initiate virus multiplication) give rise to immunity. Another possible explanation is that in individuals without previous contact with the virus, certain tissues may be unfavorable sites for optimum virus multiplication, thus presenting a barrier against further progression and dissemination.[1,2] It has been shown that with certain viruses and certain tissues the development of such constitutional barriers is related to growth and maturation of the host.[1,2] Furthermore, in mice the development of some of these barriers during the rapidly growing period may be prevented or retarded by either general malnutrition or specific dietary deficiencies of thiamin, riboflavin, and presumably other factors.[2,3]

Studies concerning the influence exerted by vitamins C and D on the course of experimental poliomyelitis have yielded various results.[4,5,6] However, more recent investigations have indicated (1), that vitamin C fails to modify the course of poliomyelitis produced in monkeys by the nasal instillation of virus,[7] and (2), that a deficiency of vitamin D is not essential for the spread of poliomyelitis virus along peripheral nerves.[8]

The possible role of a deficiency of the vitamin B complex or of one of its factors in determining whether the virus-host relationship shall take the form of paralytic or non-paralytic, abortive, or inapparent poliomyelitis deserves careful investigation. The present communication describes studies we have made in 7 patients with paralytic poliomyelitis who were tested for thiamin deficiency by the intravenous thiamin load test of Najjar and Holt.[9] The results obtained were of the same order as those found in normal children.

Procedure. All subjects had clinically typical paralytic poliomyelitis which developed while they were residing in or near Cincinnati during September or October, 1940 and 1941. The tests were performed usually within a day after admission to the hospital, so that a change of diet from that of the home can hardly be considered a factor. For 12 to 24 hr prior to the test the subjects were maintained on water and fruit juice, a regimen providing only minimal quantities of the B vitamins. The load tests were carried out as follows: following emptying of the bladder by catheterization 1 mg of thiamin hydrochloride ("Betaxin"—Winthrop) was injected intravenously. A second catheterization was carried out at the end of 4 hr to obtain the test specimen. In 3 subjects the excretion of thiamin was followed at half hour intervals after the injec-

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[1] Sabin, A. B., *Science,* 1940, **91**, 84.

[2] Sabin, A. B., *J. Ped.,* 1941, **19**, 596.

[3] Sabin, A. B., and Duffy, C. E., *Science,* 1940, **91**, 552.

[4] Jungeblut, C. W., *J. Exp. Med.,* 1937, **65**, 127.

[5] Jungeblut, C. W., *J. Exp. Med.,* 1937, **66**, 459.

[6] Toomey, J. A., *Am. J. Dis. Child.,* 1940, **60**, 548.

[7] Sabin, A. B., *J. Exp. Med.,* 1939, **69**, 507.

[8] Sabin, A. B., Ward, R., Rapoport, S., and Guest, G. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 451.

[9] Najjar, V. A., and Holt, L. E., Jr., *Bull. J. H. Hosp.,* 1940, **67**, 107.

TABLE I.
Thiamin Load Tests in Patients with Paralytic Poliomyelitis.

					μg thiamin in urine excreted during test period following intravenous inj. of 1 mg thiamin					
		Paralysis		Highest temp. on day of test (°F)	Observations in patients					Range in normal control subjects (4-hr period followng test dose)*
Subject (Sex)	Age (Yrs)	Day of	Extent		1st half hr	2nd half hr	3rd half hr	4th hr	Total 4 hrs	
C.W. (M)	15	3	Partial; arms, legs	99.9	180	74	24		278	109-236 (Avg 180)
W.J. (M)	14½	5	Complete; left leg Partial; arms, right leg	99	231				231	
T.D. (M)	14	4	Partial; right arm, legs	98.6				244	244	
G.S. (M)	7	3	Partial; right arm Complete; legs	99.5				95	95	
G.N. (M)	5	10	Partial; legs	101.2	101	74	24		199	150-246 (Avg 207)
A.G. (M)	2¼	8	Complete; left leg	99.7	139	18	7		164	
N.W. (F)	1¹⁄₁₂	4	Partial; left leg	100.5				311	311	260-338 (Avg 302)

*Najjar and Holt.[9]

tion for 1½ hr, these specimens being likewise obtained by catheterization. The urine specimens were preserved with an equal volume of 3% acetic acid and sent by air express to Baltimore. Thiamin was measured by the thiochrome method developed by Jansen[10] and modified by Hennessy and Cerecedo.[11]

Results. In Table I are given the figures for thiamin excretion in these patients, as well as figures for a series of normal controls of various age groups which were tested by Najjar and Holt[9] with this same load test. It will be noted that 6 out of the 7 poliomyelitis patients showed figures within the normal range, and the seventh patient excreted a quantity of thiamin which was only slightly below this. It would thus appear that thiamin deficiency was not a factor in the development of the paralytic form of the disease in these subjects.

Before accepting this conclusion unreservedly it is necessary to consider certain other factors which may influence thiamin load tests. It has been shown[9] that with impaired renal function a test dose of thiamin is less rapidly excreted, the peak of excretion being reached only after 2 or 3 hr, instead of during the first half hour as in the normal subject. However, the total 4-hr thiamin excretion is not affected unless the renal impairment is extreme. In these poliomyelitis patients no tests of kidney function were made, but there was no evidence, from clinical examination or urinalysis, of any renal disease and the possibility of extreme renal impairment as an interfering factor may therefore be dismissed.

Deficiencies of other B factors may at times affect thiamin load tests, producing abnormal spilling and masking the retention of thiamin which occurs in thiamin deficiency. Najjar and Holt[9] observed abnormally high thiamin excretions in pellagra and in an experimental subject who was deprived of other B factors in addition to thiamin. Inability to utilize injected thiamin under these circumstances causes a characteristic alteration of the thiamin excretion curve; although exceedingly high in the first half hour, it falls promptly to zero, in contrast to the normal subject whose excretion of thiamin remains somewhat elevated for several hours. In order to find out whether this situation was present in the poliomyelitis patients, half-hourly excretion curves were run in 3 subjects. None of them showed the sharp drop to zero after the first half hour. Specific evidence of nicotinic acid deficiency was sought in 2 other patients (G.S. and T.D.) whose urines were tested for the fluorescent factor F_2 shown by Najjar *et al.*[12] to be absent in nicotinic acid deficiency. These tests, performed on specimens obtained just before the thiamin injection, showed F_2 values of 7 and 20 units per hour respectively, values which preclude the possibility of nicotinic acid deficiency.

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[10] Jansen, B. C. P., *Rec. d. trav. chim. d. Pays-Bas*, 1936, **55**, 1046.

[11] Hennessy, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 1939, **61**, 179.

Thus, it would seem that nicotinic acid deficiency has not masked any thiamin deficiency in these paralytic patients, nor is nicotinic acid deficiency itself a prerequisite for the development of paralytic poliomyelitis.

It is possible that deficiencies of other B factors may contribute to the development of paralysis in this disease. In this connection it is of interest to call attention to recent evidence suggesting that in the nervous system of the pig certain changes attributed in other hosts to thiamin deficiency may be due to lack of other vitamin B factors such as pyridoxine and pantothenic acid.[13,14] Although the components of the B complex are usually found together in foodstuffs and the occurrence of a deficiency related to only one member of the vitamin B group is very rare,[15] it will, nevertheless, be necessary to determine the presence or absence of possible deficiencies of one or more of the other vitamin B factors before the relationship of the status of vitamin B nutrition to paralytic poliomyelitis can be completely evaluated.[16,17,18]

Summary. The results of thiamin excretion tests in 7 children having paralytic poliomyelitis did not differ in the main from those found in normal children. This finding indicates that the status of thiamin nutrition need not be a factor in determining whether infection by poliomyelitis virus shall result in the paralytic, or in one of the nonparalytic forms of the disease. Some evidence has also been obtained that nicotinic acid deficiency need not be a prerequisite for the development of the paralytic form of the disease. The role of other members of the vitamin B complex in relation to paralytic poliomyelitis remains to be investigated.

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[12] Najjar, V. A., Stein, H. J., Holt, L. E., Jr., and Kabler, C. V., *J. Clin. Invest.*, 1942, **21**, 263.

[13] Wintrobe, M. M., Stein, H. J., Miller, M. H., Follis, R. H., Jr., Najjar, V. A., and Humphreys, S., *Bull. J. H. Hosp.*, 1942, **71**, 141.

[14] Wintrobe, M. M., Miller, M. H., Follis, R. H., Jr., Stein, H. J., Mushatt, C., and Humphreys, S., *J. Nutrit.*, 1942, **24**, 345.

[15] György, P., *Ann. Rev. Biochem.* (Stanford Univ., Calif.), 1942, **11**, 309.

[16] McCormick, W. J., *Canad. Med. Assn. J.*, 1938, **38**, 260.

[17] McCormick, W. J., *Med. Rec.*, 1942, **155**, 89.

[18] Helms, K., *Med. J. Austral.*, 1941, **1**, 717.

14001

Effect of p-Aminobenzoic Acid when Added to Purified Chick Diets Deficient in Unknown Vitamins.*

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Ansbacher[1] has reported that p-aminobenzoic acid is a growth-promoting factor for the chick. The basal diet which he used was a vitamin K deficient ration consisting largely of wheat middlings and yellow corn heated at 110° for about one week. Hammond, Miller and McClure[2] found that p-aminobenzoic acid is necessary for the

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[1] Ansbacher, S., *Science*, 1941, **93**, 164.

[2] Hammond, J. C., Miller, D., and McClure, H. E., *Poultry Sci.*, 1942, **21**, 185.

normal development of the keel bones of chicks receiving diets deficient in phosphorus.

We wish to report, in this paper, studies on the growth-promoting activity of p-aminobenzoic acid and a possible mechanism for this activity.

Day-old White Leghorn chicks were divided as evenly as possible into groups of 6 and placed in electrically heated cages with raised screen bottoms. The basal diets, prepared weekly and fed *ad libitum*, are given in Table I. The two diets differ only as to the source of biotin; in one (484K) it is supplied as kidney residue[†] and in the other (486K) as a biotin concentrate (SMA Conc. 1000). We have shown that chicks receiving these diets grow slowly, feather poorly, and become anemic.[3] The addition to these diets of a factor, or factors, found in solubilized liver and in the "eluate fraction" of solubilized liver, is necessary for normal growth.

TABLE I.
Basal Diets.

	484K per 100 g	486K per 100 g
Dextrin	59	61
Casein (alcohol extracted)	18	18
Gelatin	10	10
Salts IV‡	5	5
$CaHPO_4$	1	1
Soybean oil	5	5
Kidney residue	2	
Biotin concentrate		15 γ
Cystine	0.3 g	0.3 g
Inositol	0.1 ,,	0.1 ,,
Thiamin	0.3 mg	0.3 mg
Pyridoxin	0.4 ,,	0.4 ,,
Riboflavin	0.6 ,,	0.6 ,,
Pantothenic acid	1.5 ,,	1.5 ,,
Choline	150 ,,	150 ,,
Nicotinic acid	10 ,,	10 ,,
2-methyl-1,4-naphthoquinone	500 γ	500 γ
Alpha-tocopherol	300 γ	300 γ
Vitamin A and D concentrate	3 dps/wk	3 dps/wk

‡ *J. Biol. Chem.*, 1941, **138**, 459.

The effects of various supplements added to the basal diets are summarized in Table II. The feeding of 5 mg of p-aminobenzoic acid per 100 g of diet 484K resulted in better growth and feathering than that obtained with the basal diet alone. A higher level of p-aminobenzoic acid, 10 mg per 100 g, produced a smaller growth response. The feeding of solubilized liver or the "eluate fraction" of solubilized liver gave maximum growth and normal feathers. When p-aminobenzoic acid was fed with the "eluate fraction," no further increase in growth occurred.

When the more purified diet, 486K, was used, levels of 5 mg of p-aminobenzoic acid per 100 g had no effect on growth or feathering whereas levels between 7.5 mg and 15 mg per 100 g gave noticeable responses in both growth and feather formation. Again, a higher level of p-aminobenzoic acid, 20 mg per 100 g, was not as effective as the lower level. The addition of solubilized liver or the "eluate fraction" again produced normal growth and feathering. p-Aminobenzoic acid likewise produced a growth response when 1% whole liver substance was added to the basal diet.

These results suggested that p-aminobenzoic acid was necessary for the chick and was responsible, in part, for the growth-promoting effects of solubilized liver and of the "eluate fraction." However, an assay of solubilized liver which contains the unknown factor(s) for the chick, showed that it supplies only 5.2γ of p-aminobenzoic acid per 100 g of diet when fed at 2%. When a level of p-aminobenzoic acid was fed (10γ per 100 g of diet) which approximated the level supplied by 2% solubilized liver no appreciable gain over the basal was noted.

The more purified diet (486K) was found to contain only 18γ of p-aminobenzoic acid per 100 g. This amount, although low, exceeds by 3 to 4 times the amount present in the solubilized liver, and thus it would seem that p-aminobenzoic acid is not a specific growth factor for the chick unless it is needed at very low levels.

Because of the relatively high level of p-aminobenzoic acid required to give a growth response, it was thought that this

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[†] The residue after exhaustive extraction of defatted kidney with water, 50% ethanol, and 50% methanol.

[3] Mills, R. C., Briggs, G. M., Jr., Elvehjem, C. A., and Hart, E. B., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 186.

TABLE II.
Results of Addition of Various Substances to the Basal Diets.

Diet	No. of chicks	No. dead	Avg wt at 4 weeks	Feathers 0 = very poor 100 = very good
484K only	48	8	157	34
" + 5 mg p-aminobenzoic acid per 100 g	6	0	227	60
" + 10 " " " " " "	11	0	175	50
" + 2% solubilized liver	30	0	257	93
" + "eluate fraction" ≅ 5% sol. liver	18	1	260	89
" + " " ≅ " " " + 10 mg p-aminobenzoic acid per 100 g	6	0	261	90
486K only	36	1	158	32
" + 5 mg p-aminobenzoic acid per 100 g	6	2	146	40
" + 7.5 " " " " " "	6	0	208	60
" + 10 " " " " " "	24	1	202	47
" + 12.5 " " " " " "	6	0	194	50
" + 15 " " " " " "	6	0	199	65
" + 20 " " " " " "	6	0	167	30
" + 2% sol. liver	24	0	238	89
" + "eluate fraction" ≅ 5% sol. liver	18	0	231	87
" + 1% whole liver substance	6	0	215	70
" + " " " " + 10 mg p-aminobenzoic acid per 100 g	6	0	246	85

compound causes the synthesis of the necessary unknown factor(s). The most logical manner of such production is through intestinal synthesis. To demonstrate that intestinal synthesis of the unknown factor(s) does occur in chicks receiving the basal diet (484K), an experiment was set up feeding sulfasuxidine (succinyl sulfathiazole) which diminishes the growth of certain intestinal organisms.[4] The results show (Table III) that growth and feathering on our basal diet may be retarded by at least 50% by the feeding of sulfasuxidine, indicating that intestinal synthesis of the unknown factor(s) is slowed down. That the drop in growth was not due to toxicity of the sulfasuxidine was shown by feeding it with the "eluate fraction" whereupon maximum growth was obtained.

In order to determine whether or not p-aminobenzoic acid can stimulate the growth of intestinal organisms and thereby, possibly, the synthesis of the unknown factor(s), the following *in vitro* experiment was set up: Tubes of sterile media (10 cc) containing various levels of p-aminobenzoic acid were inoculated with a mixed culture of organisms (obtained from the duodenum of chicks on the basal diet 1-4 weeks), incubated at 37° for 24-48 hr, and the amount of growth observed. The media, low in the unknown vitamin(s), has been described by Mitchell and Snell[5] for the determination of "folic acid" and was chosen for this purpose because the properties and occurrence of "folic acid"[6] are quite similar to the properties of

TABLE III.
Effect of Sulfasuxidine on Chick Growth.

Supplement to basal diet 484 K	No. of chicks	No. dead	Avg wt at 4 weeks	Feathers 0 = very poor 100 = very good
No supplement	6	0	168	30
"Eluate fraction" ≅ 5% sol. liver	6	0	243	100
½% sulfasuxidine	5	3	73	15
"Eluate fraction" ≅ 5 sol. liver + ½% sulfasuxidine	6	0	252	100

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[4] Poth, E. J., and Knotts, F. L., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 129.

[5] Mitchell, H. K., and Snell, E. E., *Univ. of Texas Publication* No. 4137, 1941, 36 and 87.

TABLE IV.
In vitro Effect of Various Levels of p-Aminobenzoic Acid on Growth and "Folic Acid" Production of Intestinal Microörganisms from Chicks.

Gamma of p-aminobenzoic acid per tube	Turbidity reading	Folic acid content§ %
0	100	.030 (.018-.048)
10	98⅔	.031 (.023-.040)
30	98	.036 (.025-.048)
40	94½	.076 (.048-.104)
80	93	.101 (.092-.118)
100	94	.092 (.068-.124)

§ The figures of folic acid content represent the percentage activity as compared to a standard of solubilized liver.

the unknown factor(s).[3,7,8] No attempt was made to determine the various kinds of organisms contained in the mixed cultures. Growth was measured by turbidity readings in the Evelyn Photoelectric Colorimeter.

Results of a typical series are shown in Table IV. It may be seen that as the amount of p-aminobenzoic acid was raised, bacterial growth increased. This is not surprising since it is known that p-aminobenzoic acid is a growth factor for various microorganisms. In this series measurements of each tube for "folic acid," according to the Mitchell and Snell method[6] showed that as growth increased the synthesis of "folic acid" increased approximately 3-fold.

The significance of these *in vitro* studies is that p-aminobenzoic acid can stimulate "folic acid" production and growth of microorganisms found in the intestine of chicks. This would indicate that other unknown factor(s) may be produced and then when p-aminobenzoic acid is *fed* to chicks on diets low in such factor(s) it may also cause the production by intestinal synthesis of the unknown factor(s). Further work must be done before definite conclusions can be drawn.

In Table II it can be seen that although the two basal diets were similar the more purified diet (486K) required a greater amount of p-aminobenzoic acid to give a growth response than did diet 484K. Assays of both kidney residue and the biotin concentrate show that neither of them contain enough p-aminobenzoic acid (supplying 0.4γ and 2.4γ per 100 g respectively) to account for this difference. However, we can explain this difference on the assumption that the kidney residue affects the intestinal flora in a different manner than the biotin concentrate, and thus changes the concentration of p-aminobenzoic acid necessary in the intestine for maximum bacterial stimulation. It is also possible that the kidney residue contains some other factor which makes the p-aminobenzoic acid more effective.

Likewise, we can now explain the report of Ansbacher[1] in which he concluded that p-aminobenzoic acid was a factor of the vitamin B complex and that it was growth-promoting for the chick. The ration which he used was no doubt low in the unknown factor(s) since Waisman *et al.*[9] have shown that heated grain rations of this type are low in these factor(s). When the high level of p-aminobenzoic acid (30 mg per 100 g) was added to Ansbacher's ration, stimulation of bacterial growth in the intestine may have occurred as it did in our experiments.

Summary. p-Aminobenzoic acid at levels of 5 to 15 mg per 100 g of diet produces growth responses in chicks receiving purified rations low in the unknown vitamins but complete in all other respects. Evidence is given however to suggest that p-aminobenzoic acid is not a specific growth factor for the chick but may act indirectly by stimulating intestinal microörganisms to produce unknown factor(s) essential for the chick.

We are indebted to Merck and Company, Rahway, N.J., for the crystalline vitamins; to Wilson Laboratories, Chicago, Ill., for solubilized liver (liver fraction L), and whole liver substance; to Wilson and Co., Inc., Chicago, Ill., for gelatin; and to Allied Mills, Inc., Peoria, Ill., for soy bean oil.

We are grateful to Mr. L. J. Teply and Mr. J. O. Lampen for the p-aminobenzoic acid assays.

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[6] Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1941, **63**, 2284.

[7] Hutchings, B. L., Bohonos, N., Hegsted, D. M., Elvehjem, C. A., and Peterson, W. H., *J. Biol. Chem.*, 1941, **140**, 681.

[8] Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

[9] Waisman, H. A., Mills, R. C., and Elvehjem, C. A., *J. Nutrition*, 1942, **24**, 187.

14002

Activation of Pro-Vitamin D_3 in the Rat.

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The experiments of Boer *et al.*,[1] who isolated 7-dehydro-cholesterol from cholesterol of animal origin were followed by the discoveries of Brockman, Drummond, Simons *et al.*,[2-4] who identified activated 7-dehydrocholesterol as the main constituent of the natural Vitamin D in fish liver oils, indicating that the Vitamin D found in the animal organism is Vitamin D_3 in contra-distinction to Vitamin D_2 which is formed by activation of ergosterol.

In view of the ability of the animal organism to form biologically highly active sterol derivatives of a hormone nature, it was considered of importance to determine whether the animal organism, *per se*, possesses the means of transforming cholesterol or cholesterol-derivatives into Vitamin D_3. Several of these compounds are now being investigated, namely, 7-keto-cholesterol, 7-hydroxy-cholesterol and 7-dehydro-cholesterol. This report concerns the fate of 7-dehydro-cholesterol in the rat.

A sample of 7-dehydro-cholesterol was prepared from cholesterol by the method of Windaus.[5] The product was repeatedly recrystallized from methyl alcohol in darkness; 0.0053 g of the purified substance was finally dissolved in 50 ml of propylene-glycol, C.P.

The activated 7-dehydro-cholesterol used in these experiments was made by irradiating an aliquot of the solution of the provitamin by pouring the solution into a Petri dish. The source of irradiation was a 2-arc therapeutic lamp held over the dish at a distance of 12 in for 30 min. The arc was held in such a position that the incident rays formed an angle of 45° with the surface of the solution.

Forty-two rats which had been on a rachitogenic diet for 21 days and which showed to be fully depleted by appearance as well as by examination of control rats were divided in groups of 7, continued on the rachitogenic diet and treated as follows:

Group I—Injected subcutaneously with 0.1 ml propylene-glycol daily and kept in total darkness throughout the experiment.

Group II—Injected subcutaneously with 0.1 ml propylene-glycol daily and irradiated daily for 4 min with a 2-arc therapeutic carbon lamp at a distance of 12 in. The animals were also daily exposed to sunshine for 3 hr.

Group III—Given daily 0.1 ml orally of propylene-glycol and irradiated as under Group II.

Group IV—Injected subcutaneously every other day with 0.2 ml of the 7-dehydro-cholesterol solution in propylene-glycol and exposed to irradiation and sunlight as under Group II.

Group V—Injected subcutaneously every other day with 0.2 ml of an *activated* 7-dehydro-cholesterol dissolved in propylene-glycol and kept in total darkness during the 8 days' experiment.

Group VI—Given orally every day 0.1 ml of activated 7-dehydro-cholesterol and kept in total darkness.

Group VII—Given subcutaneously every other day 0.2 ml of 7-dehydro-cholesterol in propylene-glycol and kept in darkness.

After 8 days all the rats were killed, the tibiæ were removed and sectioned, and the degree of calcification was evaluated by the line-test. The results are seen in Table I.

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[1] Boer, A. G., Reerink, E. H., van Wijk, A., and van Niekerk, J., *Proc. Acad. Sci. Amsterdam*, 1936, **39**, 622.

[2] Haslewood, G. A. D., and Drummond, J. C., *J. Soc. Chem. Ind.*, 1936, **55**, 598.

[3] Brockmann, H., *Ztft. Physiol. Chem.*, 1936, **241**, 104; 1937, **245**, 96.

[4] Simons, E. J. H., and Zucker, T. F., *J. Am. Chem. Soc.*, 1936, **58**, 2655.

[5] Windaus, A., Lettre, H., and Schenck, F., *Ann.*, 1935, **98**, 520.

TABLE I.

Group of 6 rats	Dosage per rat	Treatment of rats during 8-day assay period	Degree of healing observed
I	0.1 ml daily, subcutaneously, of propylene-glycol	Kept in darkness	0
II	Same	Irradiated daily	0.1
III	Same, orally	” ”	0.2
IV	0.2 ml every other day subcutan. of 7-dehydro-cholesterol in propylene-glycol	” ”	1.86
V	Same of *activated* 7-dehydro-cholesterol	Kept in darkness	3.21
VI	Same but 0.1 ml daily orally	” ” ”	3.43
VII	Same but 0.2 ml every other day subcutan.	” ” ”	0

The experiments demonstrate the following:

1—Activated 7-dehydro-cholesterol, as expected, shows a strong curative effect on rachitic rats when it is injected subcutaneously.

2—The subcutaneous injection of provitamin D to rachitic rats kept in darkness is totally ineffective.

3—The subcutaneous injection of provitamin D to depleted rats is effective only if the rats receive ample irradiation.

4—Irradiation of rachitic rats alone under the conditions of our experiments does not prove curative.

5—There is no essential difference in the curative effect of activated 7-dehydro-cholesterol whether it is given the rat subcutaneously or *per os*.

The failure of irradiation to produce any healing effect in the rachitic rat makes it doubtful whether the mammalian organism can produce any appreciable amount of provitamin D. It seems possible, therefore, that the 7-dehydro-cholesterol isolated by several authors from mammalian tissue was not synthesized in the body but was present as a result of previously ingested food containing it or was perhaps produced by oxidation of the cholesterol in the course of its preparation.[6]

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[6] Wintersteiner, O., and Bergstrom, S., *J. Biol. Chem.*, 1941, **137**, 785.

14003

Prothrombin Studies: III. Effect of Vitamin K upon Hypoprothrombinemia Induced by Dicumarol* in Man.

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The recent work of Overman, Field, Baumann and Link[1] and of Overman, Stahmann and Link[2] has established that in animals vitamin K administered in adequate dosage will counteract the hypoprothrombinemic effect of Dicumarol (3, 3′ methylene-bis[4-hydroxycoumarin]).

In the work reported in man this has not

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[*] Dicumarol is the collective trade-mark of the Wisconsin Alumni Research Foundation which controls the use thereof.

[†] Present address: Central Laboratories, General Foods Corporation, Hoboken, N.J.

[1] Overman, R. S., Field, J. B., Baumann, C. A., and Link, K. P., *J. Nutrition*, 1942, **23**, 589.

[2] Overman, R. S., Stahmann, M. A., and Link, K. P., *J. Biol. Chem.*, 1942, **145**, 155.

been demonstrated.[3,4,5] In our own clinical experience it has likewise been observed that synthetic vitamin K failed to alter the prolonged prothrombin time induced by Dicumarol administered for therapeutic purposes. An examination of the published data and of our own records revealed the fact that the quantities of synthetic vitamin K given were relatively small although within the limits recommended for treatment of hypoprothrombinemia as it occurs clinically and that at the same time the dosages of Dicumarol administered were relatively high. In view of the findings of the Wisconsin workers[1,2] it appeared desirable, in order to determine whether a similar relationship between vitamin K substitute and Dicumarol can be demonstrated in man, to use larger doses of synthetic vitamin K and the smallest effective quantities of the hypoprothrombinemic agent.

Methods. In a previous study we found the smallest single dose of Dicumarol adequate to prolong the diluted (12.5%) plasma prothrombin time in individuals with initial normal prothrombin time to be 100 mg. Six subjects showing this response were selected. Two additional cases were studied: In one the minimal effectual dose was 150 mg and in the other 200 mg. Five were males. The ages varied between 24 and 72 years. The diets of these subjects were not controlled. Since the diet was in all cases adequate it was felt that variations in the food intake did not significantly alter the results obtained in studies of such short duration. In each case the initial diluted plasma prothrombin time was normal or only slightly prolonged.

The technic used for prothrombin estimation was an extension of that described by Link and his associates.[6] In our procedure the prothrombin time of both whole and diluted (12.5%) plasma are estimated. The rationale has been set forth in a previous communication.[7] The thromboplastin used in this study was prepared from fresh rabbit lung. This yields a somewhat shorter prothrombin time than that found with rabbit brain.[8]

After the smallest effectual single dose was determined, the prothrombin time was estimated daily. On the fourth or fifth day the single dose of Dicumarol was given by mouth in sealed capsules of 50 mg each. Daily estimation of the prothrombin time was continued until the values returned to normal. After a lapse of several days the same quantity of Dicumarol was repeated and simultaneously and at regular intervals thereafter for the 2 succeeding days, synthetic vitamin K was given perorally and intramuscularly. The prothrombin time was followed for 3 days. In one case the sequence was reversed.

Results. The prothrombin times were within the normal range during the period prior to administration of Dicumarol except in Cases 4 and 5 in which there was slight initial prolongation of the diluted plasma prothrombin time.

The single dose of Dicumarol induced prolongation of the diluted plasma prothrombin time in each instance (Fig. 1). In Cases 3[‡] and 5 the whole plasma prothrombin time increased also.

After simultaneous feeding of Dicumarol and synthetic vitamin K and continuation of the latter on the succeeding 2 days the

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[3] Butt, H. R., Allen, E. V., and Bollman, J. L., *Proc. Staff Meet. Mayo Clin.*, 1941, **16**, 388.

[4] Meyer, O. O., Bingham, J. B., and Axelrod, V. H., *Am. J. Med. Soc.*, 1942, **204**, 11.

[5] Lehmann, J., *The Lancet*, 1942, **1**, 318.[§]

[§] In a subsequent communication which appeared after this paper was prepared Lehmann reports that he observed correction of the Dicumarol-induced hypoprothrombinemia by synthetic vitamin K. *Science*, 1942, **96**, 345.

[6] Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

[7] Shapiro, S., Sherwin, B., Redish, M. H., and Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 85.

[8] Shapiro, S., Redish, M. H., and Campbell, H. A., manuscript in preparation.

[‡] Attention is drawn to the fact that in Case 3, in which the minimal effectual dose was 200 mg, the diluted plasma prothrombin time and the difference between the values of the prothrombin times of whole and 12.5% plasma were less than normal. A state of hyperprothrombinemia is believed to be a factor responsible for this.[7]

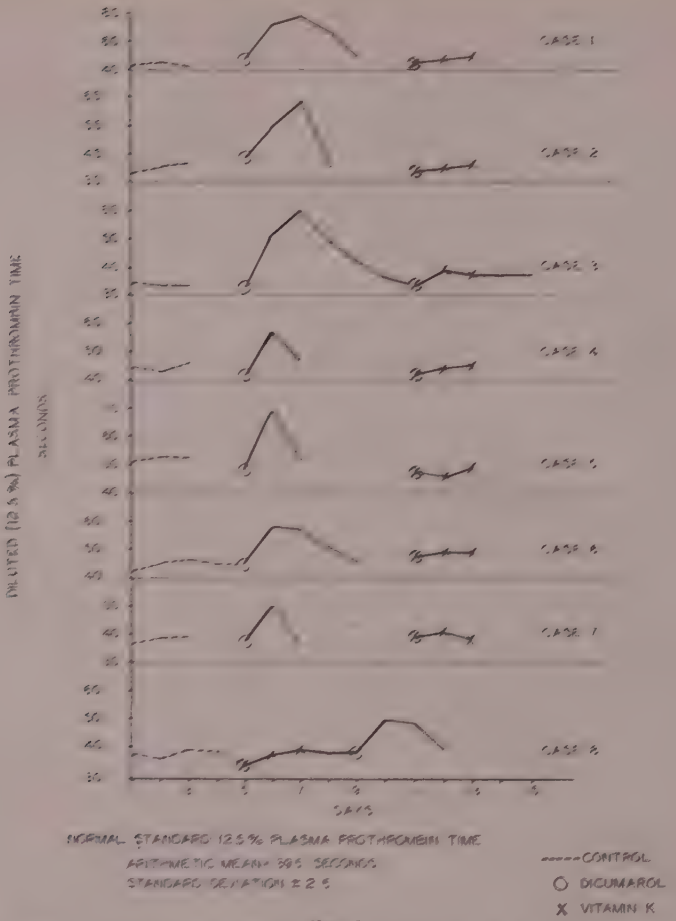

FIG. 1.

100 mg amounts of Dicumarol were given in all instances except Cases 3 and 8 where 200 mg and 150 mg respectively were administered. All doses were given orally.

Synthetic vitamin K was given as follows: 15 mg every 6 hours by mouth and 5 mg every 8 hours by intramuscular injection, making a total of 75 mg per day. In Case 3 the injections were given every 6 hours.

prothrombin times remained approximately within the range observed during the control period.

Discussion. The single dose of Dicumarol induced prolongation of the diluted plasma prothrombin time in each case. When this dose and the complementary synthetic vitamin K were administered practically no prolongation of the prothrombin time followed. This indicates that in the high levels used here synthetic vitamin K inactivated or neutralized the single minimal effectual dose of Dicumarol. That this action was not caused by the initial feedings of the hypoprothrombinemic agent was shown in Case 8 (Fig. 1) in which the order in which the experiment was conducted was reversed. This is in agreement with previous observations which show no development of sensitivity or immunity following administration of Dicumarol.

It is important to note the particulars in which the present study differs from the previous work in man referred to above.

First, much smaller amounts of the hypoprothrombinemic agent were used; and second, much larger doses of synthetic vitamin K were administered. That these were the significant differences was shown further by repeating the experiment in Case 3 using the same dosage of synthetic vitamin K and a larger dose (300 mg) of Dicumarol. Prolongation of the prothrombin time was not prevented at these respective levels.

It appears that the Dicumarol-induced hypoprothrombinemia involves a mechanism separate from that of a prothrombin deficiency consequent to a nutritional (vitamin K) lack. This is suggested by the fact that the doses ordinarily adequate for therapeutic effect do not detectably alter the prolonged prothrombin time caused by the anticoagulant although the extent of prothrombin time prolongation might be comparable.

If a quantitative relationship can be established between the activity of Dicumarol and of vitamin K it might be utilized as a means of biologically standardizing preparations of compounds exhibiting vitamin K properties. Work is now in progress to determine this.

Our findings are in accord with those of Link and his associates.[1,2]

Conclusion. In man, as has been demonstrated in animals, synthetic vitamin K administered in relatively large amounts has been found to counteract or neutralize the hypoprothrombinemia induced by a smallest single effectual dose of Dicumarol.

ACKNOWLEDGMENTS

The work was conducted in part at the Home of the Daughters of Israel, New York City, through the generous cooperation of Doctor Julius Raab.

Dicumarol and oral synthetic vitamin K (Proklot) were supplied by Dr. K. K. Chen of Eli Lilly & Co. Synthetic vitamin K for intramuscular use (Namaquin) was supplied by the Endo. Co.

Miss Frances Kaufman gave technical assistance.

The work was aided in part by a grant from the Arlington Chemical Co., Yonkers, New York.

14004

Modification of the Yeast-Growth Assay Method for Biotin.

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We wish to report a modification of the yeast-growth method of Snell *et al.*[1] for the assay of biotin in tissues and body fluids. This alteration was considered desirable since the existing method was found to be useful over a very limited range of biotin concentrations. Moreover, we encountered many instances of non-specific stimulation of yeast growth, particularly by human and canine urine samples. Our experience suggested that the medium previously employed was somewhat deficient for maximal growth of *S. cerevisiae.*

Mitchell and Williams[2] have demonstrated that certain amino-acids as well as a casein digest greatly enhance the growth of several strains of *S. cerevisiae* in a synthetic medium. These findings suggest that the addition of a biotin-free casein digest to the medium of Snell *et al.* may afford more nearly maximal growth and eliminate the occurrence of non-specific stimulation. We have accordingly developed a new medium containing an optimal amount of biotin-free casein hydrolysate.

Since our performance of the assay differs in many details from that of the earlier workers it is advisable to describe our procedure in some detail.

Method. The medium requires the following ingredients of highest purity:

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[1] Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1940, **62**, 175.

[2] Mitchell, H. K., and Williams, R. J., *Biochem. J.*, 1940, **34**, 1532.

Sucrose (Reagent)	34	g
$(NH_4)_2 \cdot SO_4$	5	"
$KH_2 \cdot PO_4$	3.4	"
d-l aspartic acid	170	mg
$MgSO_4$	.45	g
$CaCl_2$	.45	"
Mineral mixture (*cf.* Snell[1])	.18	mg equivalent
Inositol	9	"
10% biotin-free casein hydrolysate	1	g equivalent (pH 3.0)
Thiamine	1	mg
Pyridoxine	1	"
Calcium pantothenate	.5	"

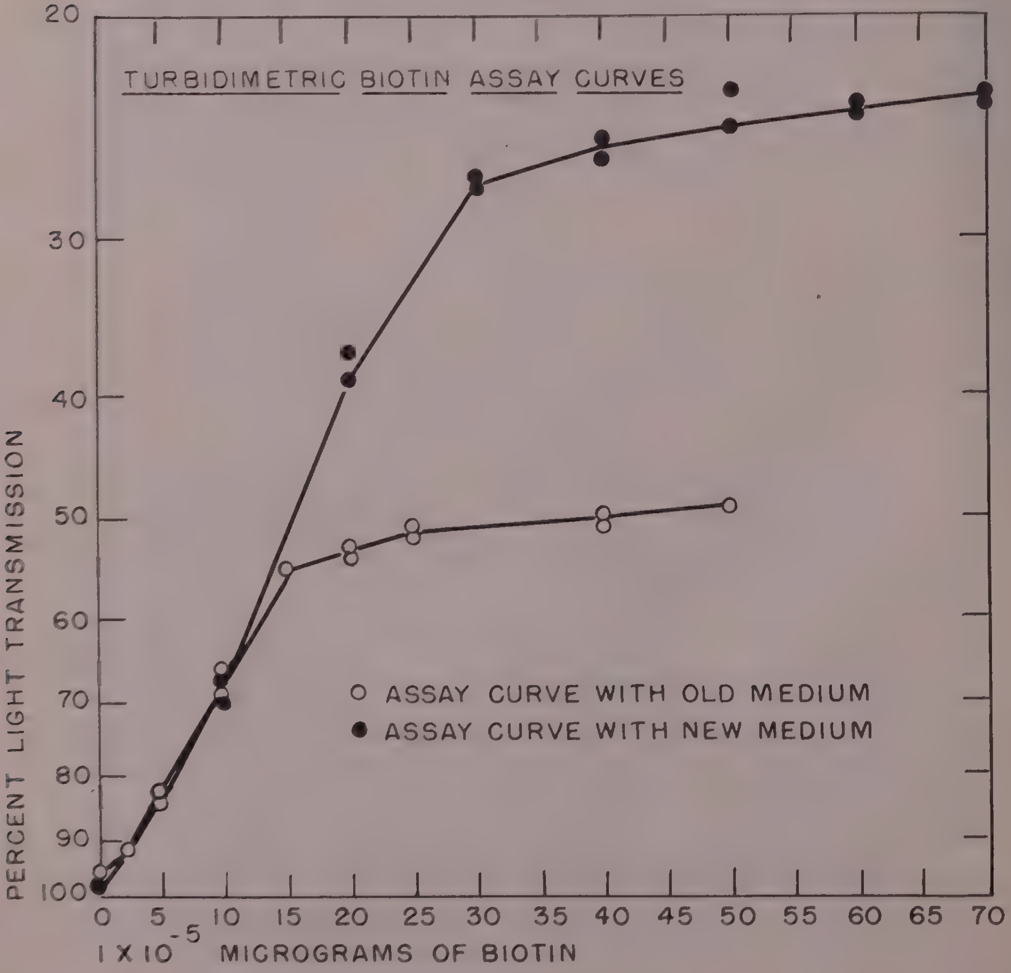
Add distilled water to make 1 liter. The pH of this preparation without adjustment is usually between 3.8 and 4.0.

These materials are added successively. Gentle warming may be required to dissolve completely. The medium may be stored in the refrigerator for at least 3 weeks without deterioration.

The test is carried out in previously calibrated photometer tubes measuring 18 cm x 2 cm. In each culture tube 5 cc of the above medium are added to variable portions of the test material and the final volume in all tubes is adjusted to 10 cc with distilled H_2O.

A stock culture of *Saccharomyces cerevisiae* (No. 139)* is carried at room temperature

FIG. I



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*Obtained through the courtesy of Doctor C. N. Frey, Fleischmann Yeast Co. Laboratories.

on Sabboraud's agar, transfers being made at least every 3 days. The test inoculum is prepared from a 24-hr culture grown at 37° in test medium to which has been added a maximally effective quantity of biotin. This culture is washed twice with normal saline and its final concentration adjusted turbidimetrically so that the .05 cc used for each test culture will contain approximately .05 mg of suspended yeast.

The test cultures are incubated without plugs for 18 to 22 hr at 37°C in a forced-draught incubator.

Assay values are determined turbidimetrically in relation to a standard curve obtained for each run in duplicate with crystalline biotin dilutions ranging in content from 1×10^{-4} to 5×10^{-4} μg per culture.

Samples may be prepared either by hot water or acid extraction, depending on the nature of the material to be tested. Only water-clear preparations may be employed. We have used this test almost exclusively for canine and human urine samples and have found direct dilution of the urine to yield satisfactory results.

Results and discussion. Fig. 1 presents a characteristic assay curve. For purpose of comparison, this figure also includes a characteristic curve obtained with the medium of Snell *et al.*[1] Some daily variation of absolute values is encountered, but the character of the curve is remarkably constant. The test is most practicable in the range from 1×10^{-4} through 3×10^{-4} μg. As the biotin concentration is increased sensitivity is rapidly lost. Hence the growth obtained with 5×10^{-4} μg of biotin is only slightly changed by further increasing the biotin concentration as much as 5-fold. Recovery tests range between 90 and 110% and successive dilutions of an unknown material check within 5%.

We have found that the following additional factors added singly or in combination to the test medium do not affect the biotin values obtained: Pimelic acid, nicotinic acid, riboflavin, adenine, guanine and uracil, choline and ethanolamine.

Considerable care must be taken to keep the age and preparation of the inoculum quite constant. We have found that variations in this regard will alter the results from day to day.

The advantages of the modified test are (1) an increased specificity due to the elimination of the non-specific stimulation which may result from the amino-acid content of test materials. (2) A wider working range which facilitates biotin determinations in completely unknown materials.†

Summary. The addition of a biotin-free casein hydrolysate to the test medium increases both the specificity and the range of biotin determinations by the yeast growth method.

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† Assays obtained for several natural materials will be presented elsewhere.

14005

Nitrogen Balance in Experimental Human Deficiencies of Methionine and Cystine.*

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The studies reported herewith form part of a program which was designed to throw light on the amino acid requirements of man. We have previously reported[1,2] that human subjects deficient in tryptophane or in lysine were unable to maintain their nitrogen balance, indicating that these amino acids were essential in human diets. Observations on male subjects on a diet deficient in arginine[3] indicated that, for the male at least, arginine is a dietary essential, normal spermatogenesis being maintained only when arginine was present in the diet.

The present report deals with observations on nitrogen balance in subjects deficient in one of the sulfur-containing amino acids—methionine and cystine. Three experiments were carried out on each deficiency, 2 of them on males and one on a female subject. Each subject was kept upon the deficient diet for a period of 24 days. This was preceded by a control period of 4 days on an identical diet supplemented with the missing amino acid. A similar control period of 8 days followed the deficiency period. As in our previous studies the experimental diet consisted of fats, sugar, and certain fruits and vegetables selected for their low nitrogen content. These fruits and vegetables supplied approximately 10% of the daily nitrogen requirement, the remainder being supplied by an amino acid mixture. The daily amount of this mixture was adjusted to the weight of the subject such that his total nitrogen intake was maintained at 0.1 g per kilo per day, both in the deficient and the control periods. The remaining portion of the diet, consisting of natural foods poor in protein was not adjusted to the weight of the subject, but for the sake of convenience in preparing the diet an identical quantity was given to each subject. This resulted in a relatively small variation in total calories with the weight of the individual (total calories varied from 2400 to 2700 per day); consequently, the lighter subjects received more calories per kilogram, a fact which must be borne in mind in interpreting the results.

The amino acid mixture deficient in methionine was prepared from an acid hydrolysate of casein by a modification of the Toennies oxidative procedure,[4] which converts methionine into the biologically inactive sulfone; the latter was removed by precipitation as the insoluble calcium salt. The resulting amino acid mixture, free from methionine, was fortified by the addition of 1.5% tryptophane to replace that destroyed in the hydrolysis; 1% cystine was also added because of the low concentration of the latter amino acid in casein. The final mixture was tested on rats and was found to be growth-promoting when supplemented with methionine.

The cystine-deficient diet was prepared by passing a neutralized acid hydrolysate of casein through a filter precoated with norit A. This has been found to selectively adsorb cystine,[5] the resulting filtrate of our

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* The expenses of this study were defrayed by grants from the Rockefeller Foundation, Merck and Company, Mead Johnson and Company and E. R. Squibb and Sons.

[1] Holt, Albanese, Brumback, Kajdi and Wangerin, Proc. Soc. Exp. Biol. and Med., 1941, **48**, 726.

[2] Albanese, Holt, Brumback, Hayes, Kajdi and Wangerin, Proc. Soc. Exp. Biol. and Med., 1941, **48**, 728.

[3] Holt, Albanese, Shettles, Kajdi and Wangerin, *Fed. Proc.*, 1942, **1**, 116.

[4] Toennies, G., *J. Biol. Chem.*, 1942, **145**, 667.

[5] Weidinger, A., *Rec. Trav. Chim. Pays Bas*, 1937, **56**, 562. Also unreported experiments of this laboratory.

product containing only 0.12% of this amino acid. The cystine-deficient mixture was fortified with 1.5% tryptophane to replace that destroyed during hydrolysis.

The control diets used in the pre- and post-deficiency periods were prepared as above described, the methionine poor diet being supplemented by 3% dl-methionine, and the cystine-poor diet by 1% l-cystine to provide a complete amino acid mixture.

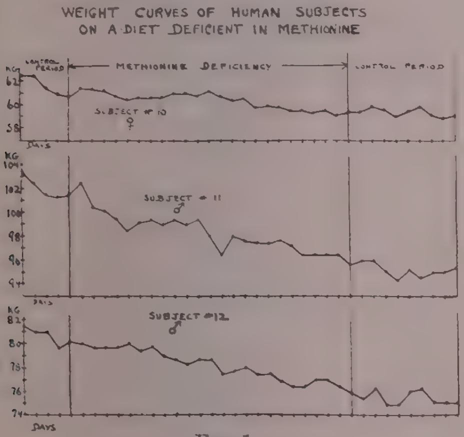

Fig. 1.

Results. The 3 subjects on the methionine-poor diet exhibited no symptoms or signs of disease during the deficiency period. They lost a small amount of weight as is shown by their weight curves (Fig. 1). Various laboratory studies were made upon them which will be reported at a later date. Only the data on nitrogen balance are presented here (Fig. 2). It will be noted that both male subjects developed negative nitrogen balances promptly after the methionine poor diet was started, and that they remained in negative nitrogen balance until the diet was supplemented with methionine. The female subject showed a minimal negative nitrogen balance during the deficiency period followed by a positive N balance when the diet containing a methionine supplement was given. In interpreting her balances we must point out that she weighed less than the male subjects and consequently received more food per kilogram of body weight. Although her nitrogen intake in terms of body weight was practically identical with that of the 2 males she received relatively more carbohydrate and fat which would have brought about some degree of protein sparing. The data on these 3 experiments indicate that methionine

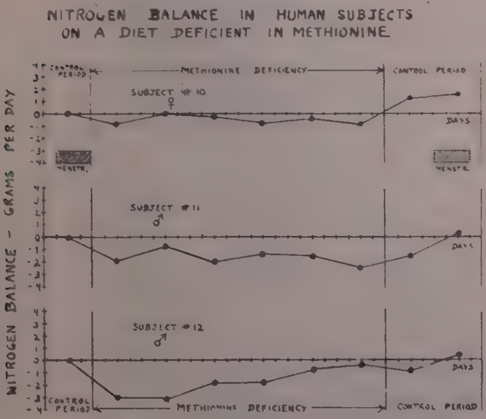

Fig. 2.

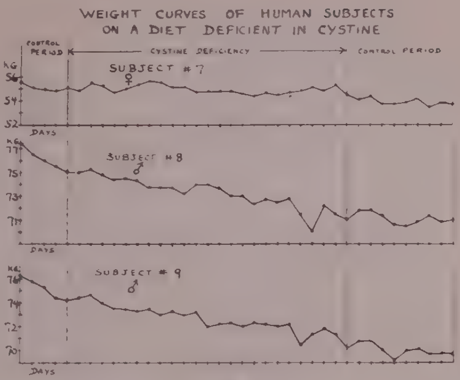

Fig. 3.

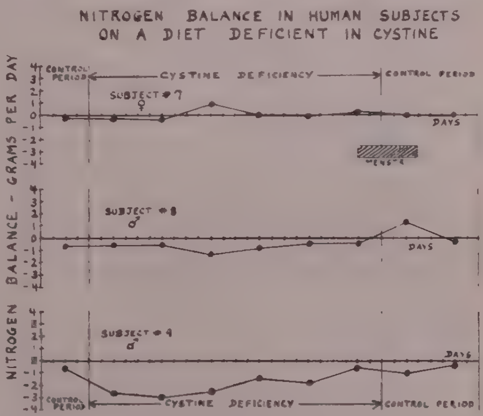

Fig. 4.

must be present in the diet in order to maintain nitrogen equilibrium and that this amino acid may be regarded as a human dietary essential.

Weight curves and nitrogen balances on 3 subjects on a cystine-deficient diet are given in Figs. 3 and 4. It will be noted that the female subject weighing 55 kg maintained her weight and remained in nitrogen equilibrium throughout the experiment. The 2 male subjects, both of whom weighed 75 kg at the outset, lost weight throughout the deficiency period. One of them showed a definite negative nitrogen balance during the deficiency period with a return to nitrogen balance in the post period when the cystine containing diet was substituted. The other subject showed only a minimal negative N balance during the deficiency with some indication of a rise in N retention in the post-period. We do not feel justified in concluding from these experiments that cystine is a human dietary essential. Although the evidence is somewhat suggestive that this may be the case, it would seem desirable to obtain confirmatory data from further experiments in which the energy quotient is constant for all subjects and protein sparing effects are eliminated.

14006

Enumeration and Differentiation of Leukocytes in the Counting Chamber with Propylene Glycol-Aqueous Stains.*

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(Introduced by C. C. Sturgis.)

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The hemolytic and solvent properties of propylene glycol suggest its use as a white blood cell diluting fluid and staining base.

Noting the occurrence of hematuria in animals following the administration of various glycols,[1,2] Von Oettingen and Jirouch[3] demonstrated that the glycols exert a hemolytic action *in vitro*. Lehman and Newman[4] in 1937 mixed propylene glycol with water, thereby discovering that all concentrations produced immediate hemolysis of rabbit blood. Similar observations with human blood were made independently by Weatherby and Haig.[5] The effect of these mixtures on the white blood cells was not mentioned.

The following observations were made by the author. Freshly drawn human blood was mixed one part in 20 parts with equal proportions of propylene glycol and water and with a concentration of 55% propylene glycol and 45% distilled water. Within 5 min the red blood cells became almost invisible, a few remained in the background but in no way interfered with the counting of the white blood cells. Leukocyte counts remained within the limits of normal variation for a period of 60 hr. (Table I.)

The mixture of propylene glycol and water in these proportions possessed many desirable features as a white blood cell diluting fluid. Its increased viscosity compared with aqueous solutions was a distinct advantage; in filling the pipette the end point was reached more accurately, there was less settling and clumping after standing in the pipette and there was less tendency to over-

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* This study was financed in part by the Parke, Davis Company.

[1] Bachem, C., *Med. Klinik.*, 1917, **13**, 7.

[2] Page, I. H., Coryllos, P., *J. Pharm. and Exp. Therap.*, 1926, **27**, 189.

[3] Von Oettingen, W. F., and Jirouch, E. A., *J. Pharm. and Exp. Therap.*, 1931, **42**, 355.

[4] Lehman, A. J., and Newman, H. W., *J. Pharm. and Exp. Therap.*, 1937, **60**, 312.

[5] Weatherby, J. H., and Haig, H. B., *J. Am. Pharmaceut. Assn.*, 1938, **27**, 466.

TABLE I.

Concentration of propylene glycol	Concentration of distilled water	30 min	2 hr	12 hr	36 hr	60 hr
50%	50%	7450	6450	6500	7200	7150
55%	45%	7250	7200	7050	7000	6850

flow the counting chamber. The background of the chamber remained clear in contrast to the debris present with acetic acid diluting fluid. The slower rate of evaporation from the chamber allowed at least 30 min for the enumeration and study of cells. When dealing with normal blood the accuracy of the total leukocyte count was not altered by standing in the pipette or by the degree of shaking.

When 0.05% eosin was dissolved in solutions containing 50 or 55% propylene glycol in distilled water the eosinophils stained brilliant red allowing their identification in the counting chamber. With 0.05% eosin and 0.05% methylene blue in the same proportions of propylene glycol and water the nuclei of all cells stained blue but the cytoplasm remained clear. The granules of the eosinophil stained rose color but allowed the blue staining nucleus to be seen through them. With low magnification (16 mm objective) the eosinophil was differentiated from other leukocytes by its color and slightly larger size. The contrast in color was intensified by increasing the light. With high magnification (4 mm objective) polymorphonuclear cells were differentiated from mononuclear cells, including lymphocytes, with sufficient clarity to allow their enumeration.

The most satisfactory results were obtained when methylene blue was dissolved in propylene glycol and later mixed with equal parts of aqueous eosin. The 2 stock solutions are made up as follows:

Solution I.

Methylene blue	0.1 g
Propylene glycol	100.0 cc

Solution II.

Eosin B[†]	0.1 g
Distilled water	100.0 cc

Mix equal parts of solutions I and II and use as the diluting fluid in the standard white blood cell pipette. The total and differential leukocyte counts are made in the standard counting chamber. A period of 3 min from the time the counting chamber is filled is required for the red blood cells to fade and for the leukocytes to settle sufficiently to count. If the chamber is filled immediately after diluting and mixing, a period of 15 min is recommended for the maximum differential staining of the white cells. A more satisfactory procedure is to allow the mixed blood to remain in the pipette for 15 min prior to filling the chamber.

It sometimes becomes necessary to filter Solution II as the eosin may precipitate on standing. The presence of a fine precipitate in the counting chamber indicates that the eosin solution requires filtering.

When made up for immediate use, filtering of the mixed stain is not essential. After standing 24 hr the mixed stain is less effective in that the outlines of the nuclei are less sharp and precipitation may occur. However, the staining of eosinophils remains unchanged.

Comparative blood studies were made in 20 normal subjects as recorded in Table II. These individuals, members of the hospital personnel, were not known to be suffering from any constitutional or local disease. Four pipettes were filled from the same puncture wound of each person. From these, 2 counts were made with the standard acetic acid diluting fluid, 2 with the stains dissolved in equal proportions of propylene glycol and water. The differential counts of 200 cells from the Wright stained coverslip, and from the direct counting chamber method, are recorded. In general there is good agreement.

Diluents with 50 and 55% propylene glycol are equally effective in determining the total leukocyte count in normal blood. Eosinophils stain well with either diluent.

[†] Eosin Y was also used and found satisfactory although it stained the eosinophil granules more lightly than eosin B. If staining is not satisfactory the eosin content may be increased.

TABLE II.
Total and Differential Leukocyte Counts by Standard Methods (Acetic Acid Diluent and Wright Stained Films) Compared with Direct Counting Chamber Determinations Using Propylene Glycol-Aqueous Stains.

Case	Total white cells				Differential white cell counts								
	Acetic acid		Propylene glycol		Wright stained smear						Propylene glycol stain		
					Polymorphonuclears	Lymphocytes	Eosinophils	Basophils	Monocytes	Smudges	Polymorphonuclears	Mononuclear cells	Eosinophils
D.F.	5450	4600	5000	5350	60.0	28.5	0.5	2.0	1.0	8.0	65.0	33.5	1.5
E.H.	5200	4900	4650	5550	61.0	36.5	1.5	0.0	0.0	1.0	66.0	33.0	1.0
A.L.	9150	8750	10900	9200	65.5	27.5	2.0	0.5	4.0	0.5	64.5	29.5	1.0
A.H.	6800	6450	6950	7100	59.5	38.0	1.0	0.0	1.0	0.5	55.5	42.5	2.0
M.W.	4050	4350	4100	3750	69.0	29.0	1.0	0.0	0.5	0.5	61.5	37.5	1.0
M.S.	3650	4250	3450	4200	50.0	47.5	0.0	1.0	1.5	0.0	51.5	47.0	1.5
H.S.	4950	5050	5300	6450	57.0	33.0	3.0	2.0	2.0	3.0	61.0	35.0	4.0
S.S.	7100	6800	7850	7000	54.5	40.0	1.0	0.5	1.0	3.0	59.0	40.5	0.5
B.C.	6750	6350	6000	6050	60.0	25.0	2.0	3.0	0.0	6.0	67.0	32.5	0.5
D.J.	6150	6400	5000	5950	63.0	27.5	5.5	0.0	0.0	4.0	65.0	32.5	2.5
E.G.	4500	4750	4750	4550	64.0	32.0	2.0	0.5	0.0	1.5	65.5	31.5	3.0
B.Y.	9450	9000	10500	9900	80.0	19.5	0.5	0.0	0.0	0.0	76.5	22.0	1.5
M.L.	6000	7350	6750	7450	70.5	24.0	1.5	1.0	0.5	2.5	79.0	21.0	0.0
T.R.	6050	5000	5250	5650	72.0	22.5	1.0	1.0	0.5	3.0	71.5	26.0	2.5
R.G.	7700	8900	7750	7000	45.0	42.5	1.0	1.0	3.0	7.5	55.0	42.0	3.0
P.W.	6300	6200	6500	5650	40.5	49.5	7.0	0.5	0.5	2.0	48.5	44.5	7.0
P.T.	9100	9250	9350	9900	68.5	24.0	2.5	2.0	0.0	3.0	74.0	21.5	4.5
J.W.	7750	7250	8050	7100	77.0	22.0	0.0	1.0	0.0	0.0	78.0	20.5	1.5
J.D.	9700	8500	8400	7900	76.5	20.0	2.0	0.5	0.0	1.0	79.5	19.0	1.5
G.P.	5750	6300	7100	7000	68.5	20.0	1.0	0.5	2.0	7.0	72.0	26.5	1.5

With 55% propylene glycol the white cell nuclei stain slightly less rapidly and intensely than with equal proportions of propylene glycol and water. When normal blood is allowed to stand in these 2 dilutions for 12 hr or longer there is a slightly greater tendency for distortion of white blood cells in 50% propylene glycol. From evidence available the higher percentage of propylene glycol may possibly prove more satisfactory in the study of abnormal blood.

The chief technical disadvantages in performing differential white blood counts from the stained film appear to be the inequal distribution of large white cells and identification of smudge cells. The former occurs particularly with eosinophils. Marked variations in the number of eosinophils between the two sides or between the center and margins of a coverslip preparation are common observations. The paucity of our present knowledge regarding the function of the eosinophil may be due in part to the inherent errors in the accepted method of counting these cells.

The routine procedure in reporting differential white blood cell counts is to ignore the unidentified, ruptured cells unless their number is relatively high. This error is also magnified by imperfections in the technic of preparing the film.

The use of the direct counting chamber differential count in conjunction with the stained film differential white cell count may result in additional evidence as to the identity of these smudge forms and may possibly control the errors of distribution resulting from variations in cell size. If this proves to be the case the actual percentage of eosinophil, polymorphonuclear, and mononuclear cells may be computed more accurately. This method is not presented as a substitute for the stained film.

Summary. A diluent of eosin and methylene blue in equal parts propylene glycol and water not only gives accurate total leukocyte counts but stains eosinophils, polymorphonuclear and mononuclear cells differentially and permits their enumeration in the counting chamber.

14007 P

Pressor Response to Acetaldehyde and Its Potentiation by Cocaine.*

Erwin E. Nelson.

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In view of the reports of the occurrence of acetaldehyde in the body and in urine, its pharmacodynamic action would seem to have merited serious investigation. Actually very little is known of its action. The only extensive study of its pharmacology is that of Handovsky, whose findings have been reported only in condensed form.[1] As pointed out by this author most of the few studies have been carried out with the use of such large doses that they have been essentially studies of the toxicology of acetaldehyde. Handovsky found that the intravenous administration of small amounts in anesthetized dogs (chloralosane) induced a sharp rise in blood pressure and a cardiac acceleration. The cardiac action was induced after removal of the stellate ganglia, ligation of the adrenals, and administration of atropine. The pressor effect was believed to be in part a direct vascular action, in part a reflex due to stimulation of chemoreceptors in the carotid body. It was suggested that acetaldehyde might be motor to non-voluntary muscle, since it was necessary only to triple the concentration normally present in blood to induce cardiac acceleration. It appears from the context that by non-voluntary muscle, Handovsky had cardiac rather than smooth muscle in mind.

The findings of Handovsky have been confirmed so far as examined, and considerably extended. Doses of the order of 5 to 15 mg per kg, given in solution in Ringer's intravenously to dogs under dial-urethane or morphine and urethane, result in a definite but transitory rise in pressure. Pithed cats and cats under dial-urethane respond similarly. The rise is frequently not quite as abrupt as that of epinephrine, but is of about the same duration. It can be obtained repeatedly, there being no evidence of tachyphylaxis. The combination of clamping both carotids and sectioning both vagi does not appreciably modify the pressor response. The same may be said of ligation of the adrenals. (Apparently Handovsky was concerned more with the cardiac action than the pressor effect in his experiments on adrenal ligation). When nicotine is given in large and repeated doses until its pressor action is lost and the blood pressure is at a low level, acetaldehyde still induces a rise. These findings indicate that most if not all of the action is peripheral and that epinephrine liberation from the adrenals is not involved. Studies to reveal the particular area of the vascular tree involved in the rise in pressure are incomplete. However, the picture is almost identical with that following injection of equi-pressor doses of epinephrine. The volume of the nose vessels and those of the kidney usually decrease, while the volume of the hind leg may increase, decrease, or first increase and then fall, resembling in detail the various effects one may get from epinephrine.[2] The differences here as in the case of the rise in pressure, are chiefly in the somewhat slower rate of development.

Since these actions resemble so closely those following sympathetic stimulation or epinephrine injection, the sympathomimetic action has been examined further. The reaction of the nictitating membrane of the cat, after acute denervation, is one of contraction to acetaldehyde. In preliminary experiments made approximately 3 weeks after removal of the superior cervical ganglion the nictitating membrane seems to have become more sensitive to acetaldehyde stimulation. Further, it has been shown clearly that cocaine increases the response to acetaldehyde in

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* Aided by a grant from the Winthrop Chemical Company.

[1] Handovsky, H., *Compt. Rend. Soc. Biol.*, 1936, **123**, 1242.

[2] Woods, G. G., Nelson, V. E., and Nelson, E. E., *J. Pharm. and Exp. Therap.*, 1932, **45**, 403.

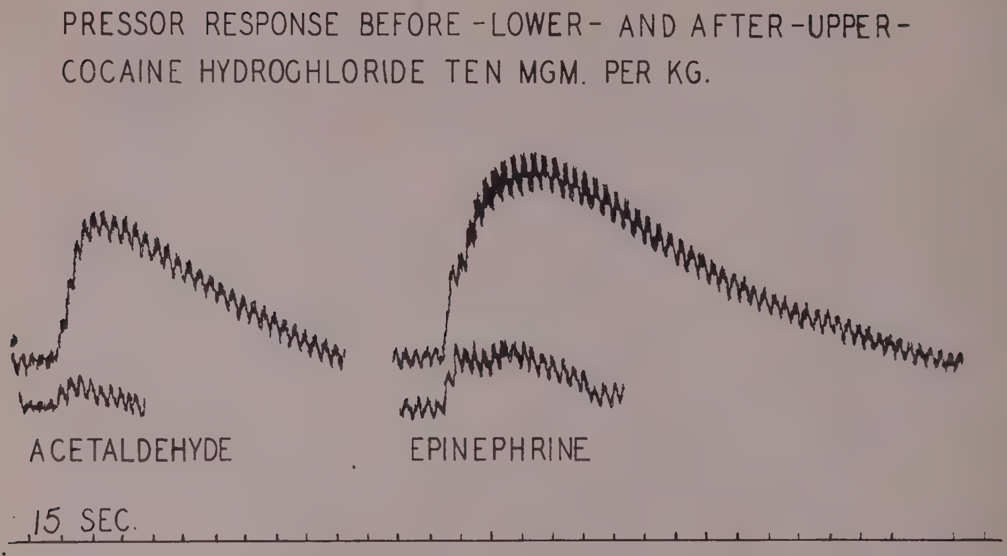

Fig. 1.

The potentiation of acetaldehyde by cocaine. Dog. Dial-Urethane. Both adrenals tied off. Each record actually started from the same level of pressure. Acetaldehyde dose 2 mg per kg. Epinephrine dose 0.00125 mg per kg. Ten minutes were allowed for absorption of the cocaine.

both acute and chronic experiments.

The most striking observation, and the one to which attention is particularly directed, is the potentiation of the pressor action by cocaine. (Fig. 1). After the intramuscular injection of cocaine hydrochloride, 10 mg per kg, this potentiation has invariably been demonstrated, regardless of other previous experimental procedures, including ligation of the adrenal vessels. The degree of effect has been quite parallel to that on control injections of epinephrine. There are obviously several mechanisms which might be involved in this response. Acetaldehyde, a very unstable substance, might persist in the blood stream in larger amounts after cocaine. Preliminary experiments on the blood concentrations reveal no differences before and after cocaine. Blood concentrations do not however necessarily give an index to the amounts diffusing into the cell. Conceivably acetaldehyde does not act *per se* but by liberation of some other substance such as epinephrine or sympathin. The adrenals as a source of the stimulant have been eliminated by ligation. Other humoral mechanisms are under investigation.

Summary. Acetaldehyde, given intravenously, has a pressor action resembling epinephrine. This action is peripheral, and resembles that of sympathetic stimulation in that it is markedly potentiated by cocaine. It is not due to liberation of epinephrine from the adrenal glands.

14008 P

Use of Adrenal Cortical Hormone in *Cl. welchii* Infections in Guinea Pigs.

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The work of Singer of Australia[1] has shown that desoxycorticosterone has a protective value in mice given lethal doses of *Cl. welchii* toxin. It is known that the adrenal glands of animals dying of *Cl. welchii* infections show congestion at autopsy.

These experiments were performed in order to ascertain whether or not relatively large doses of adrenal cortical extract† given subcutaneously to guinea pigs, which had been infected intra-muscularly with lethal doses of *Cl. welchii* organisms, would have any protective value to these infected animals.

Guinea pigs weighing between 9 and 12 ounces were used in this experiment. These animals were infected by injecting various amounts of 24-hr meat broth culture of *Cl. welchii*‡ intramuscularly, deep into the posterior muscles of the right thigh, and then crushing the muscles with a hemostat. Pigs so infected with 0.1 cc would die usually within 24 hr after the infection with the classical picture of gas gangrene infection.

Thirty guinea pigs were injected with 0.1 cc of a 24-hr broth culture, each 0.1 cc containing 16 x 10^9 bacteria (by the use of a standard bacterial counting chamber). Thirteen of these animals served as controls. Eleven animals were given 2 cc of adrenal cortical extract, each cc containing 25 dog units, one hour before infection and 6 injections of 2 cc every 4 hr thereafter. Six animals were given 3 doses of 4 cc each every 4 hr after infection.

There were 12 deaths in the controls with an average survival time of 28.8 hr. There were 6 deaths in the first group of 11 treated animals, one of which was due apparently to a traumatic atelectasis, with an average survival time of 30 hr in those animals dying from the infection. All but one of the second group of 6 animals receiving 3 doses of 4 cc each of the extract 3 died, with an average survival time of 47.5 hr. *Cl. welchii* were recovered from the thighs of all animals that died of the infection.

In another series, 10 animals were infected with 0.2 cc of a similar culture of *Cl. welchii* (24-hr meat broth culture) 8 of which were injected immediately with 2 cc of adrenal cortical extract subcutaneously and 2 cc every 4 hr thereafter until death, 2 animals serving as controls. One control died 8 hr, the other dying 12 hr after infection.

Five of the treated animals died 16 hr after infection having received in all 10 cc of adrenal cortical extract. The sixth animal died 24 hr after infection having received a total of 14 cc of adrenal cortical extract; the seventh animal died 32 hr after infection, having received a total of 18 cc of adrenal cortical extract, and the eighth animal died in 36 hr after infection, having received a total of 20 cc of the extract.

TABLE I.
Infecting Dose—0.1 cc *Cl. Welchii.*

No. of animals	Treatment cc adrenal cortical extr.	Avg survival time, hr	Survivors
13	0	28.8	1
6	3 doses of 4 cc every 4 hr	47.5	1
11	6 doses of 2 cc 1 hr before infection, then 2 cc every 4 hr	30	5

Infecting Dose—0.2 *Cl. Welchii.*

No. of animals	Treatment cc adrenal cortical extract	Survival time in hr
1	0	8
1	0	12
5	10	16
1	14	24
1	18	32
1	20	36

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* Fellow in Virus Studies and Orthopedics, National Academy of Sciences.

[1] Singer, E., *Med. J. Australia*, 1940, **2**, 275.

† Furnished by Parke Davis and Co. under the name of Eschatin.

‡ Kindly furnished by Dr. A. H. Dowdy, Rochester, New York.

All animals at autopsy showed the typical picture of *Cl. welchii* infection. Microscopic studies of the adrenal cortex of the dead animals showed hemorrhages into this area.

The results are shown in Table I.

Summary and Conclusions. Under the conditions of this experiment, it would appear that the adrenal cortical hormone extract has a protective value in delaying death in experimental *Cl. welchii* infections in guinea pigs.

We are indebted to Miss Irene Zucker of this department for her assistance in this work.

14009 P

Immunologic Studies on Experimental *Trypanosoma cruzi* Infections: I. Lysins in Blood of Infected Rats.*

NADENE DENISON. (Introduced by E. C. Faust.)
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These observations were made while comparing the effects of experimental *Trypanosoma cruzi* infection in normal rats with those obtained in animals subjected to blocking of the reticulo-endothelial system with an inert substance, trypan blue. It has been shown that mechanical blockade of the reticulo-endothelial system in bacterial infection reduces antibody formation.[1] Leishmania forms of *Trypanosoma cruzi* are frequently found in cells of the reticulo-endothelial system[2] and a related organism, *Leishmania donovani,* has been used as a biological block for these cells during certain immunological studies.[3] Production of antibodies in *Trypanosoma cruzi* infections has been previously demonstrated,[4] and further studies have since been made on various immunological aspects of the infection. When rats recover from this infection they are insusceptible to reinfection and their serum is capable of protecting others against infection.[5] Some very high titers of serum have been obtained from animals immunized against this organism[6] and agglutination and precipitation tests demonstrate a degree of species-specificity.

During the course of this work simple hanging-drop preparations were made to determine the action of blood serum from the experimental animals upon cultured *Trypanosoma cruzi* of the same strain with which the animals had originally been infected. A lot of 20 rats was separated into 3 main groups: those which were infected to serve as controls; those in which the trypanosome infection had been superimposed upon a mechanically blocked reticulo-endothelial system; those which had been previously blocked with trypan blue. The animals were all of the same age and stock and the infection produced was uniformly light.[7] Equal parts of test serum and physiologic saline suspension of washed organisms from culture were mixed on a coverslip and inverted on a depression slide. Except for one set of slides the observations were made at room temperature, and a physiological saline control was kept in all cases. Five sets of observations were made to determine the effects of (1) full strength fresh serum, (2) inactivated

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* Aided by a research grant to the Department of Tropical Medicine, Tulane University, by Eli Lilly and Co.

[1] Tuft, L., *J. Immunol.*, 1934, **27**, 63.

[2] Dias, E., *Compt. rend. Soc. Biol.*, 1932, **110**, 206.

[3] Kurotchkin, J. T., and Chung, H. L., *National Med. J. China*, 1930, **26**, 43.

[4] Chagas, E., *Nov. Reun. de la Soc. Argentina*, 1936, 1-19.

[5] Culbertson, J. T., and Kolodny, M. H., *J. Parasit.*, 1938, **24**, 83.

[6] Packchanian, A., *Pub. Health Repts.*, 1940, **55**, 2116.

[7] Kolodny, M. H., *Am. J. Hyg.*, 1940, **32**, 21.

serum, (3) incubation at body temperature, (4) various dilutions of the sera and (5) comparative effect of these sera on culture forms of *Leishmania tropica* and *Trypanosoma lewisi*.

Fresh serum from infected animals caused lysis of *Trypanosoma cruzi* taken from culture. When used in full strength, serum from blocked and infected animals caused lysis in a mean time of 88 min; while serum of control animals (which were infected only), caused lysis in 61.3 min, and the serum from uninfected animals which were blocked only, produced lysis in 153.6 min. Lysis never occurred in the physiologic saline controls, in which the organisms were mildly active even after 18 hr of observation. The serum of the animals subjected to mechanical block appeared to react more slowly. When serum had been inactivated at 56°C the organisms were immobilized by it, but lysis did not occur within the period of observation. When the preparations were kept at 37°C there was no significant change in reaction time from that at room temperature, although the reaction of the sera of the 2 infected groups of animals was slightly faster. No change at all was observed with serum from blocked uninfected animals or the physiologic saline control.

On the whole the concentration of the lysin in the sera was very low. A 1:1 dilution of the serum from animals which had been infected only caused lysis within 30 min to 1½ hr; serum from animals which had been blocked and infected, within 5 to 7 hr. A 1:1 dilution of the serum from animals which had been blocked only caused no lysis of the organism within the period of observation. A 1:5 dilution of serum produced lysis within 3 to 5 hr when taken from animals which were infected only, but required 7 or more hr when taken from those which were both infected and blocked. Serum of all 3 classes failed to produce lysis within 7 hr when a 1:10 dilution was used.

In a short series of tests using *Trypanosoma lewisi* and *Leishmania tropica* as test organisms, lysis did not occur but the motility of the organisms was rapidly impaired. The physiologic saline (control) as well as serum from all 3 groups of animals caused loss of motility of *L. tropica;* for *T. lewisi* only serum from infected and infected and blocked animals caused loss of motility of the organism.

Summary. Blood serum of animals infected with *Trypanosoma cruzi* caused lysis of this organism from cultures in less than 2 hr and, when diluted 1:5, in 3 to 5 hr. When the animals were blocked as well, the time required for lysis was increased and the lytic titer of the serum was reduced.

14010

Nature of the Activity of Sulfonamides for the Tubercle Bacillus.

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The failure of the sulfonamides to meet with any marked clinical success in the treatment of tuberculosis gives rise to speculation regarding the mode of action of the sulfonamides against the tubercle bacillus. A comparison of the *in vitro* effects of a number of sulfonamides against *Mycobacterium tuberculosis* and *Escherichia coli* indicates that the mode of action is probably identical in both cases.

Experimental. The bacteriostatic properties of a number of sulfonamides were tested against a laboratory strain of *E. coli* and a rapidly growing, non-pathogenic strain of *M. tuberculosis var. hominis* (American Type Culture Collection No. 607). For the *E. coli* tests, the compounds were serially diluted starting with 10 mg % and decreasing by half, *i.e.*, 10, 5, 2.5, etc. Each tube contained a final volume of 10 cc of syn-

TABLE I.
Acid Dissociation Values and Bacteriostatic Effect of Sulfonamides on *E. coli* and *M. tuberculosis.*

No.	Compound	pK_a*	*E. coli* min. effective conc. mols/L	*M. tuberculosis* min. effective conc. mols/L	Sulfanilamide coef.† for *E. coli*	Sulfanilamide coef.† for *M. tuberculosis*
1	Sulfanilamide	10.43	$2.1\text{x}10^{-4}$	$1.4\text{x}10^{-4}$	1.0	1.0
2	N^1-Methyl sulfanilamide	10.77	$2.7\text{x}10^{-4}$	$1.3\text{x}10^{-4}$	0.78	1.07
3	N^1-Phenyl ”	9.60	$2.5\text{x}10^{-5}$	$1.0\text{x}10^{-4}$	8.4	1.4
4	N^1-p-Tolyl ”	9.82	$4.7\text{x}10^{-5}$	$1.3\text{x}10^{-4}$	4.36	1.07
5	N^1-m-Tolyl ”	9.74	$4.7\text{x}10^{-5}$	$1.3\text{x}10^{-4}$	4.36	1.07
6	N^1-o-Tolyl ”	9.96	$9.5\text{x}10^{-5}$	$2.6\text{x}10^{-4}$	2.25	5.4
7	Sulfapyridine	8.43	$1.2\text{x}10^{-5}$	$1.2\text{x}10^{-5}$	17.5	11.6
8	4-Sulfanilamido-pyridine	8.00	$5.0\text{x}10^{-5}$	$2.5\text{x}10^{-5}$	4.2	5.2
9	2-Sulfanilamido-5-amino pyridine	8.47	$5.9\text{x}10^{-6}$	$4.7\text{x}10^{-5}$	35.6	2.9
10	Sulfadiazine	6.48	$1.0\text{x}10^{-6}$	$1.5\text{x}10^{-6}$	210.0	93.5
11	2-Sulfanilamido-imidazole	9.60	$4.2\text{x}10^{-4}$	$4.2\text{x}10^{-4}$	0.5	0.33
12	Sulfapyrazine	6.04	$1.5\text{x}10^{-6}$	$1.5\text{x}10^{-6}$	140.0	93.5
13	Sulfathiazole	7.12	$1.0\text{x}10^{-6}$	$1.5\text{x}10^{-6}$	210.0	93.5
14	2-Sulfanilamido-1,3,4-thiadiazole	4.77	$6.0\text{x}10^{-6}$	$6.0\text{x}10^{-6}$	35.0	23.4
15	2-Sulfanilamido-5-methyl-1,3,4-thiadiazole	5.45	$1.5\text{x}10^{-6}$	$2.8\text{x}10^{-6}$	140.0	50.0
16	3-Sulfanilamido-5-methyl-1,2,4-oxadiazole	4.40	$2.4\text{x}10^{-5}$	$6.0\text{x}10^{-5}$	8.5	2.3
17	Sulfanilyl cyanamide	2.92	$1.0\text{x}10^{-3}$	$4.6\text{x}10^{-4}$	0.21	0.3
18	N^1-Chloracetyl sulfanilamide	3.79	$1.0\text{x}10^{-4}$	$1.0\text{x}10^{-4}$	2.1	1.4
19	p,p′-Diamino diphenyl sulfone	—	$2.5\text{x}10^{-5}$	$6.2\text{x}10^{-6}$	8.8	22.6

$$^*pK_a = \log \frac{1}{K_a}$$

$$^\dagger\text{Sulfanilamide coefficient} = \frac{\text{Minimum effective conc. (sulfanilamide)}}{\text{Minimum effective conc. compound}}$$

thetic, inhibitor-free medium as described by MacLeod.[1] The inoculum was so designed that each tube contained approximately 100 organisms per cc initially. Presence of growth was indicated by turbidity and the lowest concentration at which stasis continued for 72 hr was taken as the minimum effective concentration.

The same drug dilution scheme was employed for *M. tuberculosis,* but Dorset's synthetic medium was used as the substrate. Test tubes with a diameter of one inch were used to provide adequate surface area for the formation of the typical pellicle of the tubercle bacillus. Appearance of this pellicle was taken as the criterion of growth, and the highest dilution at which pellicle-formation failed to take place after 5 days was considered the minimum effective concentration. The inoculum for each tube was a finely divided saline suspension containing 0.1 mg moist weight of *M. tuberculosis* No. 607. Numerous repetitions have shown that these tests are reproducible to within plus or minus one drug dilution.

Results of these tests are listed in Table I. The minimum effective concentrations of the various sulfonamides are listed for both *E. coli* and *M. tuberculosis.* Also included are the acid dissociation constants for these compounds as determined by Bell and Roblin.[2] It will be noted that the compounds which have the greatest activity against *E. coli* display a similar effect on the tubercle bacillus, and compounds with a lesser degree of activity for the colon bacillus have a correspondingly diminished effect on *M. tuberculosis.* While it might be expected that the relative order of effectiveness of the compounds would be similar for the two organisms, it is probably fortuitous that the minimum effective concentrations fall so closely together in this case. Fig. 1 graph-

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[1] MacLeod, C. M., *J. Exp. Med.,* 1940, **72,** 217.

[2] Bell, P., and Roblin, R., *J. A. C. S.,* 1942, **64,** 2905.

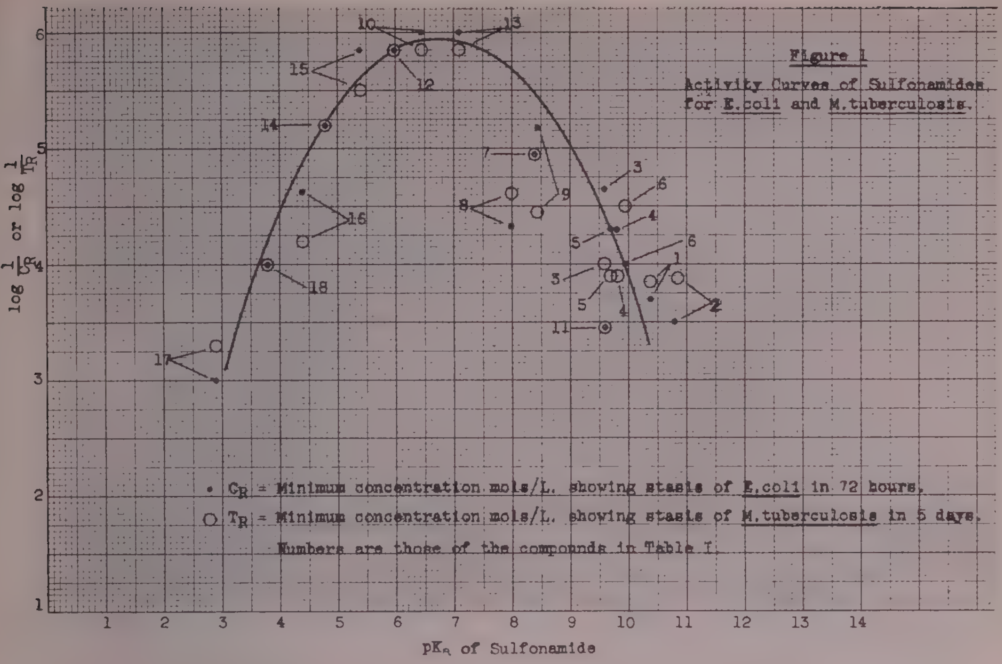

Figure 1

Activity Curves of Sulfonamides for *E.coli* and *M.tuberculosis*.

ically illustrates the relationship between the acid dissociation constants of the sulfonamides and their activity for the two organisms. The same type of activity curve is obtained in each case.

The effect of p-aminobenzoic acid on the tuberculo-static activity of several sulfonamides was studied. Varying amounts of p-aminobenzoic acid were incorporated in Dorset's medium containing varying amounts of the sulfonamides designed to cover their normal bacteriostatic range. Sodium 2,3,5-triïodobenzoate has been shown by Saz and Bernheim[3] to have some bacteriostatic activity for *M. tuberculosis*. This compound was also included here to see if p-aminobenzoic acid would have any effect on its activity. Results of this experiment are shown in Table II. The points at which growth is half inhibited are expressed as molecular ratios of the amounts of sulfonamide and p-aminobenzoic acid needed to bring about this condition. These ratios follow approximately the same order as those given by Wood,[4] Rubbo and Gillespie,[5] Rose and Fox,[6] and Wyss *et al.*[7] for *E. coli* and other organisms.

The effect of the sulfonamides on the tubercle bacillus is bacteriostatic rather than bactericidal. This could be demonstrated by the appearance of normal growth following addition of p-aminobenzoic acid to cultures that had previously undergone stasis for some time.

Discussion. It becomes apparent, from the experiments described in this paper, that compounds with a high antibacterial activity for *E. coli* may be expected to have a correspondingly high activity against *M. tuberculosis,* and the relative effectiveness is also similar for compounds which have an intermediate or low degree of activity against the colon bacillus. That is to say, while the minimum effective concentrations of the com-

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[3] Saz, A. K., and Bernheim, F., *J. Pharm. and Exp. Ther.* 1941, **73**, 78.

[4] Wood, W. B., Jr., *J. Exp. Med.*, 1942, **75**, 369.

[5] Rubbo, S. D., and Gillespie, J. N., *Nature*, 1940, **146**, 838.

[6] Rose, H. M., and Fox, C. L., *Science*, 1942, **95**, 412.

[7] Wyss, O., Grubaugh, K. K., and Schmelkes, F. C., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 618.

TABLE II.

Effect of p-Aminobenzoic Acid on Antibacterial Action of Sulfonamides on *M. tuberculosis.*

Compound	Min. effective conc. mols per liter	Sulfanilamide coef.	R_p*	R_s†
Sulfanilamide	1.4×10^{-4}	1.0	16,600	1.0
Sulfapyridine	1.2×10^{-5}	11.6	2,800	5.9
p,p′-Diaminodiphenyl sulfone	6.2×10^{-6}	22.6	332	50.0
Sulfadiazine	1.5×10^{-6}	93.5	143	116.0
Sulfathiazole	1.5×10^{-6}	93.5	106	157.0
Sodium-2,3,5-triïodobenzoate	3.8×10^{-4}	0.36	PAB has no apparent effect	

*R_p = Molecular ratio between sulfonamide and p-aminobenzoic acid at point where anti-sulfonamide activity of p-aminobenzoic acid is half inhibited.

$$\dagger R_s = \frac{R_p \text{ for sulfanilamide}}{R_p \text{ for each sulfonamide}}$$

pounds may not necessarily be identical, the relative *order* of effectiveness of a given group of sulfonamides will be similar. The observation of Bell and Roblin[2] that the potential bacteriostatic activity of a sulfonamide may be correlated with its acid dissociation constant is confirmed.

It has been shown that p-aminobenzoic acid is able to inhibit the bacteriostatic effect of the sulfonamides on *M. tuberculosis.* The amount of p-aminobenzoic acid necessary to bring about such inhibition increases with the relative bacteriostatic power of the individual sulfonamide. This would indicate that the mode of action of the sulfonamides for the tubercle bacillus is the same as for other microörganisms. These findings constitute further evidence in support of the contention of Wyss *et al.*[7] that the antibacterial activities of the sulfonamides are non-specific.

While results obtained with a non-pathogenic strain may not necessarily hold true for virulent strains of the tubercle bacillus, it is felt that such confirmation will not be lacking. The work of Smith *et al.*,[8] and the results of experiments not yet published by the present authors, indicate that the sulfonamides that have been tested follow the same general order of effectiveness for the pathogenic as for the non-pathogenic strains of *M. tuberculosis,* though the minimum effective concentrations may be somewhat higher.

The use of the non-pathogenic strain provides a rapid and simplified quantitative bacteriostatic test for *M. tuberculosis* that is especially helpful in selecting active members from large groups of compounds.

The sulfonamides have been shown to be potentially as effective against the tubercle bacillus as they are against other microorganisms. It is not inconceivable, with the advent of inherently more active compounds or the discovery of more effective means to insure that the drugs reach the organisms, that the clinical response to tubercular infections will be more marked than can be demonstrated with the sulfonamides at present.

Summary. 1. A simple method for quantitatively estimating the effect of sulfonamides on the tubercle bacillus is described. 2. The antibacterial activity of the sulfonamides for *M. tuberculosis* can be correlated with their acid dissociation constants and their ability to withstand the inhibitory action of p-aminobenzoic acid. 3. The theories of the non-specificity of action of the sulfonamides may be extended to include their effect on the tubercle bacillus.

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[8] Smith, M. I., *et al.*, *J. Pharm. and Exp. Ther.*, 1942, **74**, 163.

14011

Effect of Serum on Hemolysis by Gramicidin and Tyrocidine.

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Since two of us (Heilman and Herrell) reported on the hemolytic activity of tyrothricin[1] and its fractions, gramicidin and tyrocidine,[2] some conflicting views have appeared in the literature regarding the relative hemolytic activity of the two fractions. Experimental data from different sources are not in disagreement for the most part but differences of technic and interpretation have led to divergent conclusions.

When gramicidin or tyrocidine in minimal effective amounts is added to suspensions of washed sheep erythrocytes, the rate of hemolysis is much more rapid with tyrocidine than with gramicidin but the latter, although slower in action, ultimately causes a greater degree of hemolysis than the former. If incubation is permitted to proceed for more than 4 hr, the amount of hemolysis caused by gramicidin is greater, in our experience, than that caused by tyrocidine. It can be seen that if similar tests are done and the final readings are made after a short period of incubation, tyrocidine would appear to be much more hemolytic than gramicidin. About the same length of time is required for gramicidin to lyse erythrocytes as to destroy pneumococci.[3]

The results of studies on hemolysis by gramicidin and tyrocidine vary greatly with the kind of medium in which the tests are done. Many agents known to be hemolytic in saline solutions are ineffective in whole blood. Since hemolysis in the animal body takes place in the presence of blood serum, we have found it convenient to study the effect of various agents on rabbit erythrocytes in a tissue culture clot consisting of rabbit's plasma and serum containing a small amount of chick embryo extract. With this kind of preparation we have found that hemolysis by gramicidin takes place within an hour if relatively large amounts of the drug are used, and that smaller amounts of gramicidin require a longer period.[2] Tyrocidine, even in large amounts (0.1 mg per cubic centimeter), did not cause any hemolysis in this kind of preparation after 24 hr of incubation.

The subject appeared to be of sufficient importance to present the results of the following experiments. Sheep erythrocytes were washed 4 times with 0.85% solution of sodium chloride and were used to make 1% suspensions of erythrocytes in 0.85% solution of sodium chloride and in saline solution containing varying amounts of horse serum. The horse serum in these experiments had been kept under sterile conditions at 4°C for several weeks and had come to equilibrium with the air. The gramicidin used was prepared by Osterberg from tyrothricin according to the method of Hotchkiss and Dubos.[4] The purified tyrocidine hydrochloride, as well as the tyrothricin used in the preparation of gramicidin, was furnished through the courtesy of Sharp and Dohme. Stock solutions were prepared containing 2% of gramicidin or tyrocidine hydrochloride in 95% alcohol. Dilutions of each drug were made by the addition of varying amounts of the stock solutions to 0.85% solution of sodium chloride. To small test tubes were added 1.8 cc of the suspension of erythrocytes and 0.2 cc of a dilution of the drug being tested. The tubes were shaken and placed immediately in a water bath at 37°C. Observations on the degree of hemolysis were made after one, 2, 4, 6 and 18 hr of incuba-

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[2] Herrell, W. E., and Heilman, Dorothy, *J. Clin. Invest.*, 1941, **20**, 583.

[3] Heilman, Dorothy H., and Herrell, W. E., *Proc. Staff Meet., Mayo Clin.*, 1942, **17**, 321.

[4] Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, 1941, **141**, 155.

TABLE I.
Effect of Serum on Hemolysis by Gramicidin and Tyrocidine.
1% Sheep Erythrocytes in 0.85% Solution of Sodium Chloride.

	No serum (pH 6.9) Hrs incubation					1% serum (pH 7.1) Hrs incubation					5% serum (pH 7.7) Hrs incubation					50% serum (pH 8.25) Hrs incubation				
Gramicidin, mg per cc	1	2	4	6	18	1	2	4	6	18	1	2	4	6	18	1	2	4	6	18
.04	1*	2	4	4	4	1	1	4	4	4	0	0	3	4	4	0	0	4	4	4
.02	0	1	3	4	4	0	0	3	4	4	0	0	2	3	4	0	0	4	4	4
.004	0	0	3	4	4	0	0	1	2	4	0	0	0	1	4	0	0	0	0	4
.002	0	0	2	4	4	0	0	0	1	4	0	0	0	0	1	0	0	0	0	1
.001	0	0	1	3	4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
.0005	0	0	0	2	4	0	0	0	0	0										
Tyrocidine																				
.04	4	4	4	4	4	3	3	4	4	4	0	0	0	0	0	0	0	0	0	0
.02	2	3	4	4	4	1	1	2	2	3	0	0	0	0	0	0	0	0	0	0
.004	1	1	1	1	1	0	0	0	0	0	—	—	—	—	—	—	—	—	—	—
.002	0	0	0	0	0	0	0	0	0	0	—	—	—	—	—	—	—	—	—	—

*Degree of hemolysis, graded on basis of 1 to 4.
0—No hemolysis.

tion. Results taken from a typical experiment are presented in Table I.

The addition of only 1% of horse serum to the saline solution decreased the hemolytic activity of both gramicidin and tyrocidine. Five and 10% of horse serum caused further inhibition of the hemolytic activity of gramicidin and completely prevented hemolysis by tyrocidine. Increasing the ratio of horse serum to 20 and 50% did not result in a further decrease of the hemolytic activity of gramicidin beyond the inhibition caused by 5% of serum.

Dubos has shown that 5% dextrose will prevent hemolysis by as much as 0.4 mg per cc of gramicidin but will not prevent hemolysis by tyrocidine. Two of us (Herrell and Heilman) have also found that 5% dextrose will prevent hemolysis by relatively large amounts of gramicidin (0.1 mg per cc).[5] The data presented in similar studies by Rammelkamp and Weinstein[6] are not in agreement with the data published by Dubos or with our experimental results. They found that hemolysis of human or rabbit erythrocytes by gramicidin was not prevented or even decreased by the presence of 5% dextrose. It is well known that dextrose in a concentration of 5% protects erythrocytes from hemolysis by a number of agents. For instance, we have found that 10 μg per cc of aerosol OT (dioctyl ester of sulfonated succinic acid) will cause hemolysis within a few minutes of 1% sheep erythrocytes in 0.85% solution of sodium chloride containing 0.4% glucose, whereas increasing the amount of glucose to 2.0% delays hemolysis for 4 hr. We have shown, however, that hemolysis by gramicidin is not prevented by any concentration of glucose that would normally be present in the blood.[5]

That the inhibition of hemolysis obtained by the addition of serum is not due to the glucose present in the serum is also evident from the fact that the activity of tyrocidine is affected as well as that of gramicidin. The addition of 0.04 mg per cc of tyrocidine to the suspensions of erythrocytes used in these experiments caused a slight shift toward acidity of not more than 0.15 pH units. The same amount of gramicidin did not alter the pH of the solutions used. Both gramicidin and tyrocidine were found to be as active in causing hemolysis in Tyrode's solution at pH 8.0 as they were in 0.85% solution of sodium chloride. Therefore, the hydrogen ion concentration of the medium did not appear to be a factor in these experiments.

Summary. When gramicidin or tyrocidine in minimal effective amounts is added to suspensions of washed sheep erythrocytes, the rate of hemolysis is much more rapid with tyrocidine than with gramicidin but the latter, although slower in action, ultimately causes a greater degree of hemolysis than the former.

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Comparative experiments have been done to determine the influence of horse serum on the rate and amount of hemolysis caused by gramicidin and tyrocidine. The hemolytic activity of both substances is decreased in the presence of only 1% of horse serum. Five percent of serum causes further inhibition of the hemolytic activity of gramicidin and completely prevents hemolysis by tyrocidine. Increasing the amount of serum beyond 5% did not result in a further decrease of the hemolytic activity of gramicidin.

14012

Influence of Vitamins and Coliform Bacteria on Sulfaguanidine Tolerance by Young Chickens.*

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Sulfaguanidine is well established as an effective bacteriostatic agent for treatment of enteric disorders, but prolonged feeding of this drug in large amounts is known to cause a variety of pathological changes, including loss of weight or failure to maintain normal growth in experimental animals such as rabbits,[1] rats,[2] and chickens.[3] Black *et al.*[4,5] reported that a basic cause of growth inhibition by sulfaguanidine is its interference with intestinal bacteria which normally synthesize growth-promoting substances used by the animal body. At least some of the harmful effects of the drug can be counteracted with liver extract,[4,6] p-aminobenzoic acid,[4] vitamin K,[5] or biotin concentrate[6] but not with nicotinic acid.[7] Presumably these agents act by supplying vitamins ordinarily procured from intestinal bacteria, or by preventing the interference of sulfaguanidine with essential metabolic processes. To our knowledge no substances which increase the harmful effects of sulfaguanidine have been reported.

The wide implications and apparent acceptance of this concept of interference with synthesis by the intestinal flora led us to undertake a further investigation of the relation of vitamins and intestinal bacteria to the action of sulfaguanidine. The results obtained with young growing chickens are presented here to indicate the complexity of the relationship; they are regarded as neither an evaluation of the phenomena involved nor a criticism of previous work.

Experimental methods. Single-comb, white Leghorn chicks of uniform size and vigor were individually fed 3 times daily on an adequate ration designated as Nebraska 8-S.[8] Controlled feeding was begun 24 hr after hatching and individual records of food intake and body weight were recorded throughout the experimental period of 3 to 4 weeks.

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The following supplements were added to the ration in daily portions as required for each group of birds:

(1) Sulfaguanidine—1% of body weight of the bird per day

(2) Vitamin mixture
  (a) p-aminobenzoic acid—12 mg per bird per day
  (b) thiamin—0.6 mg per 100 g of ration
  (c) riboflavin—0.8 mg per 100 g of ration

(3) *Escherichia coli* (24-hr cultures on nutrient agar or Long's medium[9])
Water suspension of cells corresponding in turbidity to tube 8 of the McFarland[10] nephthelometer series—0.2 ml per g of ration.

Droppings were collected on clean Kraft paper placed beneath the wire mesh floor of each brooder compartment. The period of sampling was limited to 2 hr in order to provide fresh samples for laboratory use. Aliquots of moist feces were weighed and decimal dilutions to 1:10 million were then made in sterile water. Bacteriological examinations included direct microscopic counts, plate counts on enriched tryptone-glucose-cysteine agar[11] both with and without 0.045% potassium tellurite, gas production in brilliant green-bile-lactose broth[12] and coliform counts on desoxycholate agar[13] plates. Incubation at 37°C for 2-4 days was employed. Because of the extent of these data only summaries of average coliform counts are included in the tabulated results.

Results. Preliminary experiments showed that daily feeding of sulfaguanidine at levels of 3-5% of the body weight of the baby chicks caused a rapidly fatal toxicity. Lower levels of 1-2% retarded growth and caused some mortality but permitted the survival of a majority of the birds. Repeated bacteriological examinations of feces from sulfaguanidine-treated birds and controls bore out Corwin's[1] observation that the drug does not lower the total bacterial content of the feces even though there is marked inhibition of the coliform group, for a concomitant increase in Gram-positive cocci occurs. These findings indicated that 1% sulfaguanidine would give marked growth inhibition for studying the influence of selected vitamins, and that the coliform bacteria were the most likely group among the intestinal flora to play a decisive role.

Table I summarizes the data obtained from birds which received the standard 8-S ration alone and with the vitamin mixture or the *E. coli* suspension, both in the presence and absence of 1% sulfaguanidine. Repetition of each phase of this investigation 2 to 4 times yielded the same trends in every in-

TABLE I.

Relation of Sulfaguanidine, *E. coli*, and Vitamins to Survival and Fecal Coliform Counts of Young Chicks.

Supplement to 8-S ration	No. birds	No. deaths	Avg coliform bacteria/g feces × 1000
None	16	1	3,372
Vitamin mixture*	15	0	4,056
E. coli suspension	16	1	3,529
Sulfaguanidine, 1%	28	7	24
’’ ’’ + vitamin mixture*	21	12	1,847
’’ ’’ + *E. coli* suspension	21	15	242

*Thiamin, 0.6 mg/100 g ration + riboflavin, 0.8 mg/100 g ration + p-aminobenzoic acid, 12 mg per bird per day.

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stance; therefore, only total or average results are reported.

The most striking aspect of these experiments was the increased mortality rate shown by birds receiving either the vitamin mixture or the *E. coli* suspension in conjunction with sulfaguanidine. Control groups receiving 8-S ration alone and with the vitamins or bacterial suspension showed death rates of 0-6%. Those receiving only sulfaguanidine in the 8-S ration suffered a mortality rate of 25%. Addition of both the vitamins and sulfaguanidine to the ration increased the rate to 57%. A suspension of *E. coli*, recently isolated from the feces of a normal chick, combined with sulfaguanidine produced even greater harm, causing a death rate of 71%. In short the presence of a mixture of p-aminobenzoic acid, thiamin and riboflavin doubled the number of deaths resulting from administration of 1% sulfaguanidine and a suspension of *E. coli* nearly tripled the deaths from this cause.

By contrast with the above differences in mortality rate, all birds receiving sulfaguanidine alone and with supplements of vitamins or *E. coli* gained about the same amount, *i.e.*, 15 to 20 g per 100 g of feed consumed. This growth was only ⅓ of the increase maintained by chicks not receiving the drug, regardless of whether or not bacterial and vitamin supplements were included in the diet.

Counts of coliform bacteria made at weekly intervals on desoxycholate agar showed that feces of birds receiving no sulfaguanidine contained in excess of 3 million coliform bacteria per gram of moist sample. The feeding of *E. coli* did not significantly increase the number present in the droppings but the vitamin mixture did tend to give higher counts. Addition of sulfaguanidine to the 8-S ration caused progressive dimunition of these bacteria until practically none were present after 3 weeks. In the presence of the vitamin mixture or the *E. coli* suspension the drug decreased the coliform bacteria, but appreciable numbers persisted throughout the period of observation.

Conclusions. The death rate among baby chicks fed an adequate ration plus sulfaguanidine at the level of 1 g per 100 g body weight per day is less than half that occurring when a mixture of p-aminobenzoic acid, thiamin and riboflavin or a suspension of *E. coli* are administered with the drug. These facts, together with the tendency of both the vitamins and bacterial suspension to sustain the coliform bacteria in the feces in the presence of an inhibitory concentration of the drug, suggest that the tolerance of sulfaguanidine by young birds may be related to the activities of the coliform bacteria in the intestinal tract.

Our findings do not, in themselves, constitute a contra-indication to the clinical use of sulfaguanidine, for the level of drug employed here is much in excess of the effective therapeutic dosage. They serve only to emphasize the apparently fundamental and complex relationship of vitamins and intestinal bacteria to the action of sulfaguanidine *in vivo*.

The authors wish to express their appreciation to Lederle Laboratories, Inc., for supplying sulfaguanidine, to Prof. F. E. Mussehl, Chairman of the Department of Poultry Husbandry, for furnishing chicks and 8-S ration, and to Professors Geo. L. Peltier, C. W. Ackerson and R. M. Sandstedt for making possible the interdepartmental cooperation necessary for this investigation.

14013

Amino Acid Requirement of *Lactobacillus casei.*

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During recent years the lactic acid bacterium, *Lactobacillus casei,* has been studied rather extensively with respect to its growth factor requirements. From these studies it has been established that the essential growth factors needed by this organism are: riboflavin,[1] pantothenic acid,[2] nicotinic acid,[2] pyridoxine,[3] biotin,[4] and the eluate factor (folic acid).[3,4,5] The effect of stimulatory factors, that is, factors that the organism can synthesize slowly but which, if added preformed to the medium facilitate growth during early periods, have not as yet been studied in such detail. Glutamine, asparagine, adenine, and guanine have been reported, under certain conditions, as falling into this category.[6]

Recently it was reported that *L. casei* could be subcultured on a medium containing riboflavin, pantothenic acid, nicotinic acid, pyridoxine, biotin, concentrates of the eluate factor, glucose, minerals and acid hydrolyzed casein as a source of amino acids.[4] It is the purpose of this communication to describe the amino acid requirements of *L. casei* on this defined medium.

Method of assay. Assay procedure followed accepted technics. The inoculum was prepared by transferring the organism from a stock culture into a medium of the following composition:

	%
Glucose	2.0
Sodium acetate	0.6
Acid hydrolyzed casein	0.5
Tryptophane	0.01
Cystine	0.01
	mg %
Adenine	2.0
Riboflavin	0.02
Calcium pantothenate	0.02
Nicotinic acid	0.02
Pyridoxine	0.02
Biotin (free acid)	0.00001
Eluate factor concentrate	0.001

Salts per 1000 ml of medium as follows:
K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot 4H_2O$, 0.01 g.

After 24 hr incubation at 37°, the cells were centrifuged down and resuspended in 10 ml of sterile 0.9% saline. One ml of this suspension was diluted to 10 ml with sterile saline. One drop of this suspension was

TABLE I.
Effect of Omitting One Amino Acid on Growth.

Amino acid omitted from basal medium*	ml of N/10 acid per 10 ml of medium
None	7.8
Glycine	7.5
dl Alanine	5.8
dl Leucine	0.5
dl Isoleucine	5.2
dl Norleucine	7.4
dl Serine	0.6
dl Threonine	6.7
dl Phenylalanine	0.7
dl Lysine	6.8
dl Methionine	6.6
dl Glutamic acid	0.4
dl Valine	0.5
dl Aspartic acid	0.5
l Cystine	0.4
d Arginine	0.5
l Histidine	5.3
l Tryptophane	0.8
l Proline	7.5
l Hydroxyproline	7.4
l Tyrosine	0.7

*The basal contained all of the constituents listed on this page excepting acid hydrolyzed casein. This was replaced by a mixture of the 20 amino acids given above; 1 mg of each amino acid per 10 ml of medium.

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[1] Snell, E. E. Strong, F. M., and Peterson, W. H., *Biochem. J.,* 1937, **31**, 1789.

[2] Snell, E. E., Strong, F. M., and Peterson, W. H., *J. Am. Chem. Soc.,* 1938, **60**, 2825.

[3] Snell, E. E., and Peterson, W. H., *J. Bact.,* 1941, **39**, 273.

[4] Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.,* 1941, **141**, 521.

[5] Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.,* 1941, **63**, 2284.

[6] Feeney, R. E., and Strong, F. M., *J. Am. Chem. Soc.,* 1942, **64**, 881.

added per assay tube. The assay tubes were incubated at 37°. At convenient intervals during the first 30 hr of growth turbidity readings were made with an Evelyn photoelectric colorimeter and at the end of 72 hr incubation the amount of lactic acid formed was determined by titration. As the same relative results were obtained by both methods only the titration values are given. All experiments were run at least 3 times and in some cases 5 times.

In the amino acid experiments, the acid hydrolyzed casein, tryptophane and cystine were replaced by a mixture of 20 amino acids. In the first part of the work (Table I) the level of each amino acid was 1 mg per 10 ml of medium. Later it was found that this concentration was too low to insure good growth and the level was raised to 2 mg per 10 ml of medium (Tables II and III). At this concentration the organism grew just as well as when hydrolyzed casein supplemented with tryptophane and cystine was used as the source of amino acids.

TABLE II.
Effect of Stimulatory Amino Acids.

Amino acid added to basal medium	ml N/10 acid per 10 ml of medium
Basal*	3.2
Threonine	6.3
Threonine + methionine	7.4
20 amino acids	10.8
Hydrolyzed casein	10.9

*Besides glucose, buffer, adenine, vitamins and salts the basal contained leucine, serine, phenylalanine, glutamic acid, valine, aspartic acid, cystine, arginine, tryptophane and tyrosine; 2 mg of each amino acid per 10 ml of medium.

TABLE III.
Antagonistic Relations Among Amino Acids.

Amino acid added to basal medium	ml N/10 acid per 10 ml of medium
Basal*	5.9
Histidine	5.3
Isoleucine	5.3
Lysine	6.6
Alanine	7.6
Histidine + isoleucine	4.9
Histidine + lysine	5.9
Histidine + alanine	7.1
Isoleucine + lysine	5.4
Isoleucine + alanine	7.7
Lysine + alanine	9.6
Histidine + isoleucine + lysine	5.4
Histidine + isoleucine + alanine	7.2
Histidine + lysine + alanine	9.6
Isoleucine + lysine + alanine	11.2
Histidine + isoleucine + lysine + alanine	11.5
Hydrolyzed casein	11.4

*Contained the ten amino acids listed in the footnote of Table II plus threonine and methionine; 2 mg of each amino acid per 10 ml of medium.

The effects of omitting each of the 20 amino acids in turn from the medium but retaining the other 19 are summarized for a typical experiment in Table I. No growth or only slight growth occurred in the absence of leucine, serine, phenylalanine, glutamic acid, valine, aspartic acid, cystine, arginine, tryptophane and tyrosine. Growth was restricted in the absence of alanine, isoleucine, threonine, lysine, methionine and histidine. The need for cystine and tryptophane confirmed the previous report[1] that these 2 amino acids are required by *Lactobacillus casei.*

In the next experiment the effect of adding threonine and methionine to a basal containing the 10 amino acids found to be essential in the previous experiment was determined. The data are given in Table II. With all 10 essential amino acids poor acid production was obtained. Improvement resulted when threonine and methionine were added but the acidity was not equal to that obtainable with 20 amino acids. Experiments were therefore set up to determine how many more amino acids were needed to reach the top level.

When the other stimulatory amino acids were added singly to the above 12 amino acids, certain antagonistic relations were observed. A typical series is given in Table III. Histidine and isoleucine were inhibitory while lysine and alanine were stimulatory. Furthermore, the inhibition of histidine and isoleucine and the stimulation of alanine and lysine were somewhat additive. Lysine counteracted the inhibitory effect of histidine and of isoleucine. Alanine more than reversed the effect of histidine or isoleucine either alone or of the two in combination. Isoleucine, lysine and alanine together produced a marked stimulation and the addition of histidine, making a total of 16 amino acids, increased the growth to that obtained with hydrolyzed casein. The growth on the basal was not equal to that obtained with the same combination in Table II. Variations occurred from series to series but the relative results within a given series were the same for 5 different trials. Such variations are likely to occur in sub-optimal media and are probably related to the vigor of the organism in a given inoculum and its ability to overcome an unfavorable environment.

Summary. The amino acid requirement of *Lactobacillus casei* was determined on a medium containing glucose, sodium acetate, salts, riboflavin, pantothenic acid, nicotinic acid, biotin, pyridoxine, adenine, and concentrates of the eluate factor (folic acid). The simplest amino acid mixture that would yield maximum growth on repeated subculture was: leucine, serine, phenylalanine, glutamic acid, valine, aspartic acid, cystine, arginine, tryptophane, tyrosine threonine, methionine, alanine, isoleucine, lysine, and histidine.

14014

Effect of Salt and Substrate Concentration upon Activity of Adenosinetriphosphatase.*

JOHN W. MEHL AND EDWIN L. SEXTON.

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Although the procedure used by some workers for determining adenosinetriphosphatase (ATP-ase) activity[1,2] is adequate for many purposes, such a measurement of the increase in inorganic phosphate at a single time is not necessarily proportional to the enzyme activity. The present studies were undertaken to determine under what conditions the initial rate of formation of inorganic phosphate would be linear, or could be taken as proportional to the enzyme concentration. In the course of the study, it also became evident that the activity of the enzyme was markedly affected by the salt concentration. These observations will also be reported.

Materials. The adenosinetriphosphate (ATP) was prepared from rabbit muscle by the method of Kerr.[3] The dibarium salt of ATP was precipitated twice from water, and was dried over P_2O_5. Although the amount of organic phosphorus was close to the theoretical value, the labile phosphate (readily hydrolyzed with 1 N acid at 100°, and corresponding to 2 moles of phosphate per mole of ATP) indicated that the material was only about 60% pure. The concentrations of ATP given are calculated from the labile phosphate.

For use in the experiments, a weighed amount of the dibarium salt was dissolved in 0.1N HCl and the barium precipitated by the addition of a slight excess of solid Na_2SO_4. The barium sulfate was removed by centrifugation or filtration, the solution neutralized with NaOH, and made up to volume.

The ATP-ase was prepared from rat muscle by the procedure commonly used for the preparation of myosin. Muscle from the hind limb was frozen in dry ice, ground in a Wiley mill, and extracted with 0.5 N KCl. The pH was kept in the neighborhood of 7 to 7.5 during the extraction by the addition of $NaHCO_3$. The extract was centrifuged at a speed of 10,000 RPM in an angle centrifuge. The pH of the supernatant was adjusted to 6.5 with acetic acid, and 7 volumes of cold water added. The myosin precipitate was centrifuged off and washed once with cold water. The myosin was then dissolved with sufficient KCl to make the concentration 0.5 M. The precipitation was repeated twice more, and the myosin was kept in 0.5 M KCl

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* This work was aided by a grant from the Rockefeller Foundation.

[1] Needham, D. M., *Biochem. J.*, 1942, **36**, 113.

[2] Bailey, Kenneth, *Biochem. J.*, 1942, **36**, 121.

[3] Kerr, Stanley E., *J. Biol. Chem.*, 1941, **139**, 121.

till used. All of the steps in the myosin preparation were carried out in the cold room.

Methods. The measurements were made by adding all of the components of the solution except the enzyme and adjusting the volume with water so that the final volume, after the addition of the myosin, would be 10 ml. The experiments were carried out in test tubes in a water bath at 30 ± 1°C. A sample was taken 20 to 30 sec after the addition of the enzyme, and at intervals of 5, 10 and 15 min. In each case a 2 ml sample was removed with a pipette and delivered into 2 ml of 10% trichloracetic acid. The concentration of inorganic phosphate was determined by the method of Bodansky,[4] using a Beckmann spectrophotometer.

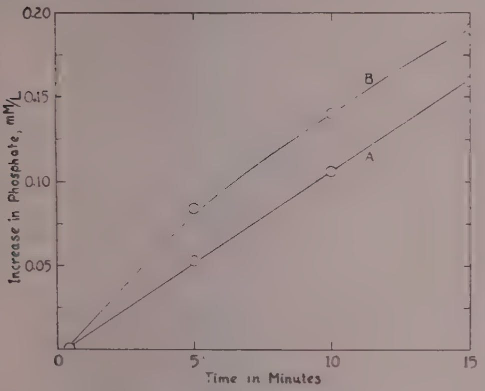

FIG. 1.

Initial rate of increase of inorganic phosphate. 0.45 mM ATP, pH 7.35. Curve A, 0.1 M glycine, 0.123 g myosin per liter. Curve B, no glycine, 0.205 g myosin per liter.

In all cases the solutions contained 0.001 M $CaCl_2$, which has been shown to be necessary for optimum activity of the enzyme.[2] The buffers which were used were diethylbarbituric acid, $NaHCO_3$ and Na_2CO_3, or glycine.

Influence of ATP concentration. Bailey's results show that, within the proper range of concentrations, the increase in inorganic phosphate in 5 min is proportional to the myosin concentration.[2] We have confirmed this finding, but were more particularly interested in determining within what range of concentration the rate of formation of inorganic phosphate would be independent of the ATP concentration.

In this connection it may be pointed out that the evidence from the shapes of the curves is not conclusive. Fig. 1 gives the results of 2 typical experiments in which the ATP concentration was 0.45 mM. Although the inorganic phosphate increases in a linear manner with time in the solution containing glycine, the increase is not linear in the absence of glycine.

Experiments were therefore carried out in which the initial rate of formation of inorganic phosphate was determined when the ATP concentration was varied. The results of measurements at pH 7.35, in the absence of glycine are given in Fig. 2. It is seen that the rate appears to vary with the ATP concentration, but not by an amount comparable with the changes in ATP concentration. In this particular set of experiments, the concentrations of the stock solutions necessitated a smaller salt concentration as the ATP concentration was increased. We had not anticipated a marked effect of salt as long as sufficient salt was present to keep the myosin in solution, but subsequent experiments in-

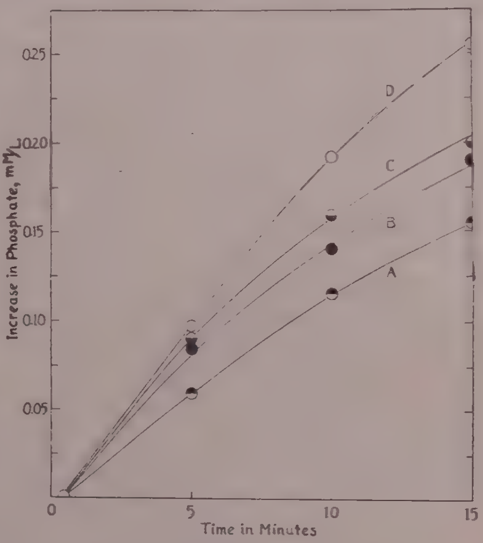

FIG. 2.

Effect of ATP concentration. 0.205 g myosin per liter, pH 7.35, no glycine. ATP concentrations 0.23, 0.45, 0.90, and 1.35 mM in curves A, B, C, and D.

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[4] Bodansky, A., *J. Biol. Chem.*, 1932, **99**, 197.

dicated a rather marked effect.

Effect of salt concentration. Experiments were carried out at pH 6.6 and pH 9.1, both without glycine and with 0.1 M glycine. The ATP concentration was 0.45 mM in all cases. The salt concentration was varied by changing the concentration of KCl. The results were calculated as activities relative to an activity of 1 at a total salt concentration of 0.55 M. It will be seen in Fig. 3 that at pH 6.6, in the absence of glycine (curve C), the activity increases about 2.5 times as the salt concentration is decreased from 0.5 M to 0.15 M. In the presence of 0.1 M glycine (curve D), the effect is somewhat less marked. At pH 9.1 the effect is less than at the lower pH, but the effect of glycine (curve B) is again to reduce the effect of salt below that in the absence of glycine (curve A).

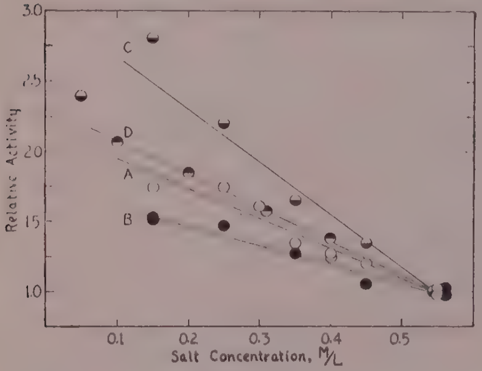

FIG. 3.

Effect of salt concentration. Curve A, no glycine, 0.45 mM ATP, pH 9.1; Curve B, 0.1 M glycine, 0.45 mM ATP, pH 9.1; Curve C, no glycine, 0.45 mM ATP, pH 6.6; Curve D, 0.1 M glycine, 0.45 mM ATP pH 6.6; Activity relative to unit activity at a total salt concentration of 0.55 M.

It is also worth noting that there is an obvious precipitation of myosin at the lower salt concentrations and pH, but this does not appear to reduce the rate of splitting of phosphate in these experiments.

In Table I are given the results of the experiments in which the ATP concentration was varied, and for which the initial rate of formation of phosphate is corrected for the salt concentration. In all cases the rate has been calculated for a total concentration of 0.5 M salt, except that the concentration of sodium glycinate has not been taken into account. Although the accuracy of the results leaves much to be desired, they do not give any indication that the initial rate is increased by increasing the ATP concentration above 0.23 mM, when the myosin (or ATP-ase) concentration is in the neighborhood of 0.1 to 0.2 g per liter.

Summary. Measurement of the activity of ATP-ase in salt concentrations from 0.15 to 0.55 M show that the activity is decreased as the KCl concentration increases. The magnitude of the effect depends upon the pH and the presence or absence of glycine. The rate of formation of inorganic phosphate is also found to be independent of the substrate concentration when the ATP concentration is 0.23 mM per liter or greater. Observed deviations from a linear rate of formation of phosphate at the higher ATP concentrations must be attributed to inactivation of the enzyme during the course of the experiment.

TABLE I.

Effect of ATP Concentration.

Myosin g/L	ATP mM/L	Glycine M/L	KCl M/L	NaCl M/L	Buffer M/L	Total salt M/L	pH	γ P per hour	γ P per hour, corr.
.205	.23	0	.35	.05	Barbiturate, 0.005	.40	7.35	255	210
”	.45	0	.30	”	”	.35	”	336	242
”	.90	0	.20	”	”	.25	”	300	220
”	1.35	0	.05	”	”	.10	”	390	194
.123	.23	0.1	.25	.05	”	.30	7.25	210	155
”	.45	”	.20	”	”	.25	”	210	146
”	.90	”	.10	”	”	.15	”	264	163
.123	.23	0.1	.50	0	Glycine	.50	9.15	378	378
”	.45	”	”	0	”	”	”	437	437
”	.90	”	”	0	”	”	”	384	384

14015

Dermatitis on a Synthetic Ration Adequate for Growth and Reproduction in the Rat.

BENJAMIN H. ERSHOFF. (Introduced by H. J. Deuel, Jr.)

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The importance of pantothenic acid, pyridoxine, and the essential fatty acids for the prevention of dermatitis in the rat has been demonstrated by a number of investigators.[1-5] It is the purpose of the present communication to point out that in addition to the above still another dietary factor(s) is involved in the maintenance of normal tail epithelium.

Procedure and results. The basal ration consisted of sucrose 73.2, Vitamin Test Casein* 22, 1(-) cystine 0.3, and Sure's Salt Mixture No. 1 4.5.† To each kg of the above were added 5 mg thiamine hydrochloride, 5 mg pyridoxine, 10 mg riboflavin, 100 mg nicotinic acid, 100 mg calcium pantothenate, 150 mg para-aminobenzoic acid, 400 mg inositol, and 1 g choline chloride. Daily supplements of 150 mg corn oil (Mazola), 0.25 mg alpha-tocopherol, and an A-D concentrate‡ equivalent to 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D were also administered.

Twenty-eight male and female albino rats of the Wistar strain were weaned at 21 days of age, and litter mates divided into the following two groups: (1) maintained on the synthetic ration listed above, and (2) maintained on a natural food ration supplemented once weekly with lettuce. Both males and females were bred at approximately 70 days of age. Females were placed with males of proven fertility, and pregnancy dated from the finding of sperm in the vaginal tract. Males, in turn, were bred to females maintained on a natural food ration adequate for growth, reproduction and lactation.

Growth. Both male and female rats on the synthetic diet were consistently heavier than litter mates receiving the natural food ration. Of 7 males raised from weaning on the synthetic diet, 5 were heavier than the largest natural food control. Similar results were obtained for females. At 100 days of age, males on the synthetic ration averaged 264 g as contrasted to 233 g for litter mates maintained on a natural food ration.

Reproduction. Five out of 7 males raised from weaning on the synthetic diet mated successfully the first time they were placed with estrus females at approximately 70 days of age. In every instance, litters of apparently normal young were born. Females raised from weaning on the synthetic diet exhibited regular estrous cycles as determined by vaginal smears. Four females were bred to normal males of proven fertility, and pregnancy dated from the finding of sperm in the vaginal tract. Young of normal birth weight were delivered at term, 2 litters of 11 each, and one of 8, that could not be distinguished from those born of natural food controls. A fourth litter consisting of 11 young of normal birth weight included 7 young born dead. In no instance did the mother nurse her young.

Dermatitis. Although the synthetic ration was adequate for promoting growth and reproduction in the albino rat, animals maintained on this ration exhibited a dermatitis

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[1] Henderson, L. M., McIntire, J. M., Waisman, H. A., and Elvehjem, C. A., *J. Nutrition,* 1942, **23**, 47.

[2] Gyorgy, P., Poling, C. E., and Subbarow, Y., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 738.

[3] Richardson, L. R., and Hogan, A. G., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 583.

[4] Salmon, W. D., *J. Biol. Chem.,* 1941, **140**, cix, Proc.

[5] Richardson, L. R., Hogan, A. G., and Itschner, K. I., *Missouri Agr. Exp. Sta. Res. Bull.,* 1941, **33**, 2.

* S.M.A. Corporation, Chagrin Falls, Ohio.

† Sure, Barnett, *J. Nutrition,* 1941, **22**, 499.

‡ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units vitamin A and 80,000 U.S.P. units vitamin D per gram.

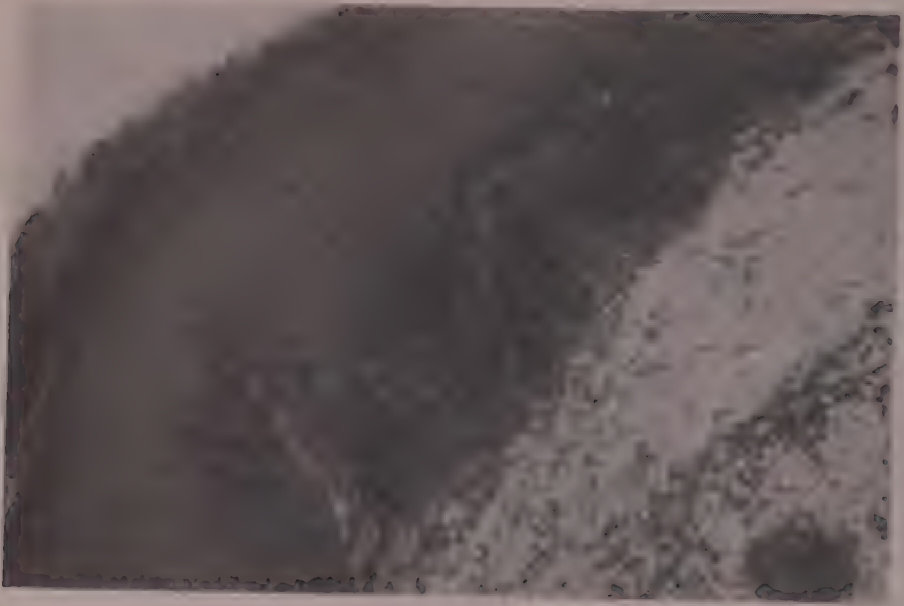

FIG. 1.
Tail of rat maintained on synthetic ration. Magnification 100

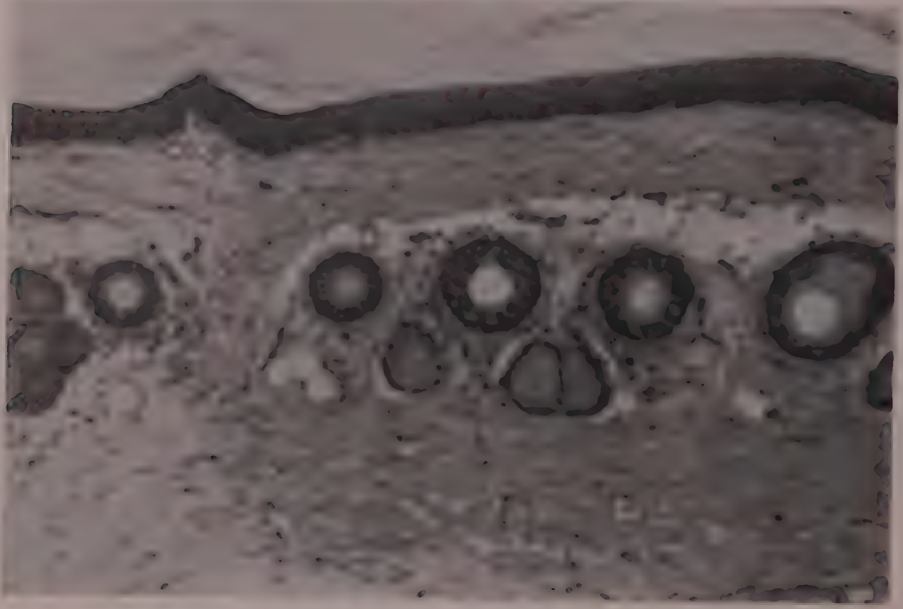

FIG. 2.
Tail of rat maintained on natural food ration. Magnification 100 ×.

of the tail that differed from that observed in pantothenic acid deficiency, pyridoxine deficiency, or deficiency of the essential fatty acids. During the sixth week on the synthetic diet, small reddish brown nodules appeared as intermittent elevations along the surface of the tail, being more prominent in the proximal than the distal portion. Histologically the pathological picture approached that of an early granulomatous papule, with a hyperplasia of the epidermis to 4 or 5 times normal thickness, a marked irregularity of the basement membrane, and a leucocytic infiltration of subepidermal tissues particularly around the basement membrane and the epithelium of the hair follicles. (Figs. 1 and 2.) By the tenth week, approximately one-fourth of the animals exhibited small areas along the cheeks and lower jaw where fur had fallen out and hard reddish-brown scabs were visible. Superficially, however, with the exception of the tail lesions, the animals could not be differentiated from litter mate controls maintained on a natural food ration.

Attempts to cure the dermatitis with a single dietary supplement have been unsuccessful. Subcutaneous administration of a biotin concentrate§ equivalent to 0.5 γ per day for a 3-week period had no effect on the alleviation of these symptoms. Similarly a solubilized liver extract rich in a factor necessary for the growth of *Lactobacillus casei*|| was also ineffective when incorporated as 2% of the ration for a period of one month. Administration of a natural food ration however resulted in a prompt remission of the dermatitis.

An interesting observation was the effect of alterations in the basal ration on the appearance of dermatitis. Fifteen litter mates of the above series (8 males and 7 females) were placed at weaning on a synthetic ration identical to the above but minus the corn oil. The sole source of essential fatty acids in the diet was accordingly that present in the purified casein. On this ration containing a total fat content, exclusive of the unsaponifiable vitamin A-D concentrate and alpha-tocopherol, of 0.04%, no evidence of scaly skin or caudal necrosis was observed, although other evidence of essential fatty acid deficiency was present: retardation of growth; poor ovulation and delayed parturition in the female; sterility in the male. From the fourth to the seventh week on this fat free diet tail lesions were observed identical to those described for the basal ration; subsequent to the seventh week however, a gradual disappearance of dermatitis occurred. At 100 days of age tails of animals on the fat deficient diet were practically symptom free in contrast to the marked dermatitis observed in litter mates receiving an identical diet supplemented with corn oil.

In a subsequent series 16 albino rats of the Wistar strain were weaned at 25 to 30 days of age and divided into the following two groups: (1) consisting of 6 rats maintained on the basal ration listed above, and (2) consisting of 10 rats (5 males and 5 females) receiving a synthetic ration identical to that of Group I but deficient in inositol. At 100 days of age animals receiving the basal ration (Group I) exhibited a dermatitis of the tail indistinguishable from that observed in the previous experiment. Animals maintained on the inositol deficient diet (Group II) were free of dermatitis.

Inasmuch as sufficient pantothenic acid, pyridoxine, and essential fatty acids were present in the basal ration to insure a rate of growth not inferior to that observed in natural food controls, and inasmuch as both males and females were able to undergo apparently normal reproduction on the synthetic diet, it is felt that some dietary factor(s) other than the above is necessary for the prevention of dermatitis. Further experiments on the identity of this factor are now in progress.

Summary. A specific tail dermatitis is described distinct from that observed in pantothenic acid, pyridoxine or essential fatty acid deficiency. It is suggested that some dietary factor or factors other than the above is involved in the maintenance of normal tail epithelium.

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§ Biotin Concentrate No. 1000, S.M.A. Corporation, Chagrin Falls, Ohio.

|| Solubilized Liver Extract Fraction L, Wilson Laboratories, Chicago, Ill.

14016

Pyridoxine Deficiency in Turkeys.

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The effects of pyridoxine deficiency have been described for the rat,[1] dog,[2] pig,[3] and chick.[4-7] In order to find whether the turkey would respond to pyridoxine deficiency in a similar manner to the chicken and the rat, poults were fed deficient and standard diets.

Deficient diet. The pyridoxine-deficient diet was similar to that reported in a previous publication.[6] However, it differed in that extracted liver and alfalfa were included each at a level of 5%.

The alfalfa meal was prepared in the same manner as the wheat bran.[6]

Beef liver was passed through a meat grinder, suspended in water, and allowed to autolyze 2 days under benzene at 37°C. The suspension was then treated with live steam until all protein was coagulated. The solids were separated in a wine press, suspended in hot water, and repressed. This was repeated twice. The solids were then dried. The combined filtrates were treated 5 times with fuller's earth, concentrated at reduced pressure, and added back to the dried residue.

Methods. Bronze turkey poults were weighed at hatching, placed in electrically heated wire battery brooders with raised wire floors, and fed a regular turkey starting mash for different periods of time, as shown in Table I. At the start of each test the birds were reweighed and segregated into groups of similar weights and gains. Experimental diets and water were given *ad libitum.* In 2 of these tests the regular all-mash ration fed by the Division of Poultry Husbandry to turkeys during the first few weeks after they are hatched was used as the only positive control. In the third test, 3 mg of pyridoxine per kg of basal diet and the regular turkey starting mash were both used as positive controls.

Results. Poults receiving the deficient diet appeared weak and apathetic, although they were hyperexcitable when disturbed. Some of them would dash around the cage bumping into any object in their path. Jerky uncon-

TABLE I.

Test	Diet	Days on normal* diet, No.	Poults started, No.	Poults finished, No.	Convulsions observed in poults, No.	Gain in weight	
						Grams	% Normal
I	Basal	4	11	2	3	32.4	12.5
	Normal*	25	10	9	0	256.7	100.0
II	Basal	11	11	7	3	43.9	11.9
	Normal*	32	10	9	0	368.8	100.0
III	Basal	6	10	3	3	20.0	6.3
	Basal + 3.0 mg pyridoxine per kg	6	10	10	0	270.5	85.2
	Normal*	28	10	9	0	317.5	100.0

*Normal = regular all-mash ration.

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[1] Chick, H., El Sadr, M. M., and Worden, A. N., *J. Biochem.,* 1940, **34**, 594.

[2] Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *J. Nutrition,* 1938, **16**, 197.

[3] Chick, H., Macrae, T. F., Martin, A. J. P., and Martin, C. J., *J. Biochem.,* 1938, **32**, 2207.

[4] Carter, C. W., and O'Brien, J. R., Proc. 7th World's Poultry Congress, Cleveland, 1939, p. 126.

[5] Jukes, T. H., Proc. Soc. Exp. Biol. Med., 1939, **42**, 180.

[6] Lepkovsky, S., and Kratzer, F. H., *J. Nutrition,* 1942, **24**, 515.

[7] Mills, R. C., Hegsted, D. M., Elvehjem, C. A., and Hart, E. B., *Poultry Sci.,* 1942, **21**, 379.

trollable movements of the head and neck were observed. Convulsions were observed in 9 out of 32 poults and largely resembled those already reported for the chick.[6]

Two convulsions in each of 3 poults followed a somewhat different pattern prior to death. These resembled the convulsions seen in acute strychnine poisoning. At the start the poults were in a comatose state lying on their sides. A fine tremor of the feet was noticed which increased in severity. Suddenly the neck would arch until the head touched the back, the legs assumed extreme extension, and the whole body became rigid and in opisthotonos. Respiratory movements ceased during such an attack. After an interval of approximately one-half minute, the poult would relax and respiratory movements would begin. After these seizures the poults were completely exhausted. In each case the poult died during the second observed convulsion. Several poults on the deficient diet were found dead with this characteristic curve of the neck with the head arched toward the back. They may have died in convulsions but such were not observed. None of these convulsions could be elicited but appeared spontaneously. These observations indicate that the effects of pyridoxine deficiency on poults are similar to those reported for chicks.[6] In turkeys, convulsions are followed by death much more quickly than in chicks.

Poults receiving the basal diet plus 3.0 mg pyridoxine per kilo of diet were normal in appearance and grew at a rate which approximated 85% of the growth of poults on the turkey starting mash.

Summary. Pyridoxine deficiency in turkeys is characterized by loss of appetite, poor growth, apathy, hyperexcitability when disturbed, convulsions, and death. Pyridoxine prevented the deficiency symptoms.

14017

Further Studies on Properties of the Gastric Secretory Depressant in Gastric Juice.*

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A gastric secretory depressant has been observed in acid gastric juice of both humans and dogs. In patients with achlorhydria and gastric carcinoma or pernicious anemia, this factor is present in relatively high concentration.[1] This factor is contained in the flocculent precipitate obtained by addition of 4 volumes of absolute alcohol to neutralized gastric juice. About 1 g of precipitate obtains from a liter of juice. Three hundred to 500 mg of the precipitate, suspended in saline and injected intravenously into gastric pouch dogs fed meat, affords consistently an achlorhydria of the pouch lasting at least 2½ hr.

Alkalinization of a suspension of the precipitate results in solution of the latter, which however reprecipitates with acidification (pH 5 to 6). This second precipitate is inactive as far as the gastric secretory depressant is concerned, as is also the supernatant fluid, hence alkalinization destroys the depressant factor. Five hundred mg of active precipitate were suspended in 20 cc of N/10 N HCl and U.S.P. pepsin added to .2% concentration. After incubation for 24 hr at 37°C no decrease in the potency of the gastric secretory depressant factor was noted. Longer periods of incubation resulted in decreased potency of the factor.

The question is raised if the gastric secre-

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* This work was conducted under a grant from the International Cancer Research Foundation, Philadelphia, Penn.

[1] Brunschwig, A., Schmitz, R. L., Rasmussen, R., *J. Nat. Cancer Inst.*, 1941, **1**, 481.

tory depressant factor in gastric juice is identical with that factor described as "urogastrone" and obtained from the urine. Gray[2] reported the consistent recovery of this gastric secretory depressant factor from urine by precipitation with benzoic acid, extraction with acid-aqueous-acetone and reprecipitation with alcohol-aniline-acetone.

One gram quantities of 80% alcohol precipitates of human gastric juice, 300 to 500 mg of which, as stated, always produced prolonged achlorhydrias in the stimulated gastric pouch, were extracted twice with 20 cc of 70% acid acetone (1 cc concentrated HCl per 100 cc solution) and these combined. The latter were then treated with one volume of absolute alcohol, 3 cc of aniline and 3 volumes of absolute acetone (Gray's method); this afforded a flocculent precipitate which was separated by centrifugation and dried. This precipitate weighing about 300 mg was found to be soluble in slightly acidified normal saline solution, and when injected into pouched dogs failed to produce achlorhydria on 8 separate occasions.

The residue of the original alcohol precipitate of gastric juice which was insoluble in 70% acid acetone was suspended in 20 cc of slightly acidified normal saline and injected into pouched dogs. In each of 6 separate experiments achlorhydria of the stimulated pouch, lasting over 2½ hr, obtained.

Conclusions. A method for extraction of the gastric secretory depressant factor from urine (urogastrone) does not extract the gastric secretory depressant from an alcohol precipitate of human gastric juice, hence evidence is offered that these two factors are not identical.

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[2] Gray, J. S., *et al.*, *Endocrinology*, 1942, **30**, 129.

14018 P

Observations on the Intravenous Injection of Gelatin for Nutritional Purposes.*

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In view of the recent interest in parenteral injection of nitrogenous nutriment, the question of nutritive value of gelatin injected intravenously is being investigated. Observations of this type apparently have not been hitherto recorded. Such a study is complicated by the heterogeneous nature of gelatin. The sample employed in this study was designated as 3 Cu, and consisted of a light brown moderately coarse powder. Aqueous solutions were prepared by adding 120 to 125 g to 1000 cc of hot water. This was autoclaved at 15 pounds pressure and 250°F for 45 min prior to injections in animals. Total nitrogen determinations were made on each sample and this data employed to calculate nitrogen intake. Non-protein nitrogen varied widely in the several liter samples prepared, the maximum in one sample being 570 mg % but in most others it was under 100 mg %. This gelatin was found to contain no cystein or tryptophane and .8% tyrosin (analyses by Mr. Carter Johnston, Department of Biochemistry).

Dogs depleted of protein stores by maintenance on a Weech diet[1] for about one month received 150 cc of the gelatin solution (which was approximately 12% by weight) a day intravenously as the sole source of protein. During the injection period Weech diet was afforded *ad libitum* but the appetite varied widely. Urine (and feces) was collected every 24 hr and the daily loss of

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* This work was conducted under a grant from the Edible Gelatin Manufacturers' Research Society.

[1] Weech, A., *et al.*, *J. Exp. Med.*, 1935, **61**, 299. (505 g of diet contains 1.23 g N.)

TABLE I.
Nitrogen Balance in Dogs Receiving Intravenous Injections of Gelatin.

	No inj.			Inj. period*				
Dog	No. days	N loss, g	Avg daily N loss, g	No. days	Total N inj., g	Total N loss, g	Avg daily N bal., g	N balance total period, g
352 (4.3 kg)				7	18.5	10.17	+1.2	+ 8.33
354 (6.4 kg)								
Exp. I	8	4.58	.57	8	27.9	14.32	+1.69	+13.58
Exp. II	5	2.92	.58	7	18.5	17.25	+ .18	+ 1.25
357 (5.6 kg)	7	4.77	.68	10	28.00	30.58	— .26	— 2.58
358 (5.6 kg)								
Exp. I	5	3.38	.68	15	41.70	41.30	+ .03	+ .40
Exp. II	5	1.28	.26	7	18.30	11.02	+1.04	+ 7.28

* Each dog received 150 cc of 12% gelatin solution daily.

nitrogen determined. This was compared with nitrogen intake as represented by the gelatin injections. Results are summarized in Table I. In 5 of the 6 experiments a positive nitrogen balance or a nitrogen equilibrium was obtained; in one instance there was a negative balance. In the latter (Dog 357) however, the daily net loss of nitrogen was less than in the period during which no injections of gelatin were received.

The fact that a positive nitrogen balance or equilibrium was obtained, does not indicate necessarily a utilization of gelatin for body or plasma protein synthesis or for energy. At least some gelatin remains unchanged and circulates in the plasma since samples of blood withdrawn 24 hr after an injection gel when placed in the ice box, and after several daily injections, some samples of urine also geled at 8°C. On the other hand it would appear that some of the gelatin is broken down by the organism since, as shown in Tables I and III, after daily injections were begun the nitrogen output exhibited a substantial increase and this was for the most part in the form of non-protein nitrogen.

When 2, 2.5 and 5% aqueous solutions of gelatin were analyzed as "blood plasma" for plasma proteins by the semi-micro Kjeldahl

TABLE II.
Plasma Protein Changes Resulting from Intravenous Injection of Gelatin.

Dog		Hematocrit, %	N. P. N., mg %	Plasma prot., g %	A/G
352	Before injection	44.5	18.2	4.52	2.09/2.43
	After 6 injections*	32	50	6.54	2.25/4.29
	4 days after last injection	38	28	5.54	2.03/3.51
	8 days after last injection	32	22	4.93	2.56/2.33
354	Before injection	37	17.5	4.73	2.65/2.08
	After 9 injections	30	26	6.10	1.52/4.58
	7 days after last injection	32	17	5.35	1.68/3.67
358	Before injection	48	23	4.22	2.41/1.81
	After 14 injections	23	—	5.79	2.65/3.14
	7 days after last injection	30	34	5.18	2.23/2.95
	14 days after last injection	28	26	4.30	2.22/2.08
356	Before injection	40	21	3.87	1.56/2.31
	After 5 injections	25	32	4.88	1.84/3.04
353	Before injection	49	17	3.90	2.15/1.75
	After 3 injections	38	322‡	8.63	2.71/5.92

*Daily injection of 150 cc of 12% gelatin solution.
‡Dog was uremic, died 24 hours after last injection.

TABLE III.
Showing Increased Urinary N Output During Period of Intravenous Injection with Gelatin. Dog 358. On Weech Diet. (Partial data.)

	No injections				Daily inj. 150 cc gelatin solution 6th to 20th day				No injection after 20th day			
Days	1	2	3	4	7	8	9	10	21	22	23	24
Urine vol., cc	280	240	250	160	240	380	400	300	200	100	140	120
Total N excreted, g	.47	.61	.56	.44	2.90	3.71	3.98	2.03	1.81	.76	1.06	.75
Total N.P.N. excreted, g					2.88	3.61	3.80	1.93	1.66	.68	.96	.65

method for total nitrogen, and precipitation with 21% sodium sulphite carried out as for separation of albumin from globulin, it was found that most of the gelatin precipitated as "globulin." For example, a 2.1% aqueous solution of gelatin yielded .68% "albumin" and 1.41% "globulin." A 5.33% solution of gelatin yielded 1.72% "albumin" and 3.61% "globulin." In another series of experiments gelatin was added to dog plasma low in proteins and the greater part of the gelatin was precipitated with the globulin fraction.

In the protein depleted dogs, the injections of gelatin resulted in a rather rapid and appreciable rise in plasma protein, this being in a large measure due to the presence of circulating gelatin. From the above mentioned observations it would therefore appear that the increase in "plasma proteins" thus brought about would constitute essentially an increase in the "globulins." Some of the data illustrating this is shown in Table II. Following the cessation of injections the nitrogen output fell appreciably and there was a gradual fall in the plasma protein level, the decrease being again principally the globulin fraction with relatively less fluctuation in the albumin fraction (with exception of Dog 354).

The animals tolerated the injections quite well. Occasionally there was emesis; in one animal marked erythema about the head and over the extremities developed after each injection. Severe reactions were not observed. Elevations in temperature were likewise not observed. Since the animals were depleted of proteins, being on a very low protein diet, they were not in good condition at the onset of the experiments. Throughout the injection period this diet was available in unlimited quantities but as stated they ate poorly and, although maintained for periods in positive nitrogen balance or in nitrogen equilibrium, they continued to lose weight and their condition deteriorated. The latter would be expected from maintenance on an incomplete diet.

Summary. 1. A positive nitrogen balance or nitrogen equilibrium may be maintained in protein-depleted dogs with a gelatin solution injected intravenously as practically the sole source of nitrogen.

2. Elevation of depressed blood plasma protein levels may obtain from intravenously administered gelatin as practically the sole source of nitrogen. The increase is apparently in the globulin fraction and represents to a large extent circulating gelatin. It was found that most of the gelatin used above in aqueous solution, or if added to plasma *in vitro*, precipitates with 21% sodium sulphite (thus behaving like a "globulin").

3. At least a portion of intravenously injected gelatin appears to be metabolized since there is a substantial increase in non-protein nitrogen excretion following injections.

14019 P

Comparison of Erythrocyte Numbers in Normal and Hormone-Treated Brown Leghorn Fowl.*

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This investigation includes a comparison of the number of erythrocytes found in the blood of normal brown Leghorn hens, roosters, capons and sinistrally and bilaterally ovariectomized poulards, with the number found following treatment with various sex hormones.

The normal male has a red cell count of 3,600,000 per cubic millimeter (average of 18) which is approximately a million higher than that of the female—2,700,000 (average of 18). The capon (18), sinistrally ovariectomized poulard (19), and bilaterally ovariectomized poulard (12) have counts of 2,510,000, 3,000,000, and 2,520,000 respectively.[1]

Since there is a decided sex difference in erythrocyte number, and since the characteristic male number is reduced in the capon whose count is equivalent to that of a bilaterally ovariectomized poulard, there appears to be a sex hormonal control of erythropoiesis, with the male hormone causing an increase in red cells and the female hormone a decrease.

To test this assumption, females, capons, and sinistrally and bilaterally ovariectomized poulards were given daily subcutaneous injections of testosterone propionate. In every case there was a rise in the erythrocyte number after 15 injections and the counts made after 24 injections were equal to or approaching that of a normal male. (See Table I). Daily injections are being continued on 6 of the 9 females shown in Table I and the high red cell numbers have now been maintained for more than 160 days.

In a similar manner injections of alpha-estradiol benzoate or diethylstilbestrol were given males, capons, and bilaterally ovariectomized poulards. With no exception, the erythrocyte counts were reduced. (See Table I). In both androgen and estrogen injected birds the red cell numbers had returned to approximately the normal levels when counts were made 21 or 45 days after injections were discontinued.

It has been shown that gonadotropic hormones injected into young birds stimulate a precocious production of hormones by the immature gonads. That androgen as well as estrogen is produced by the stimulated ovary is shown by the masculinization of head furnishings and development of oviducts in treated females.[2] Juhn and Domm[3] reported that the erythrocyte level in juvenile birds is the same as that of the mature female. In order to obtain information on the erythrocyte picture in birds treated with gonadotropins we injected a P.M.S. gonadotropic hormone into both males and females, beginning on the fourth day after hatching. In both sexes the erythrocyte levels of treated birds were above those for untreated controls. (See Table I).

Counts made on a series of 36 intersexual

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[1] Taber, E., Davis, David E., and Domm, L. V., *Anat. Rec.*, 1941, **81**, 89.

[2] Domm, L. V., *Cold Spring Harbor Symposia on Quant. Biol.*, 1937, **5**, 241.

[3] Juhn, M., and Domm, L. V., *Am. J. Phys.*, 1930, **94**, 656.

TABLE I.
Effect of Hormones on Erythrocyte Number.

No. of birds	Sex*	Treatment	Red cells in millions per mm³		
			Normal	11-15 days	24-29 days
9	F	0.50-1.50 mg testost.	2.70 (avg of 18)	3.29	3.17
6	C	1.00-3.75 " "	2.51 " " "	2.84	3.30
3	S.P.	1.25-3.75 " "	3.00 (" " 19)	3.36	3.77
3	B.P.	1.25-3.75 " "	2.52 (" " 12)	2.93	3.34
8	C	0.33-1.50 mg α-estra.	2.51 (" " 18)	2.12	2.36
3	C	1.00-2.00 " stilb.	" " " "	2.08	—
3	B.P.	0.50-1.50 " α-estra.	2.52 (" " 12)	—	1.93
2	M	2.50-5.00 " stilb.	3.60 (" " 18)	—	1.42
6	J.M.	140 I.U. P.M.S.	2.27 (" " 3)	—	2.76
6	J.F.	" " "	2.48 (" " 8)	—	3.01
3	C	0.33 mg α-estra. + 1.00 mg testost.	2.51 (" " 18)	2.99	3.30
3	C	1.00 mg stilb. + 1.00 mg testost.	" " " "	3.01	2.78

*C, capon; F, female; J.F., juvenile female; J.M., juvenile male; M, male; S.P. and B.P., sinistrally and bilaterally ovariectomized poulards.

males,[4] feminized by estrogen injection during incubation, averaged 2,850,000, essentially that of the normal female. There was no correlation between the number of erythrocytes and the degree of plumage feminization.

In an attempt to reproduce the intersexual condition experimentally, capons were given daily injections of both testosterone propionate and alpha-estradiol benzoate or diethylstilbestrol. As a result of the α-estradiol testosterone combination the average red cell count of 3 capons was raised from 2,640,000 to 3,300,000 after 25 injections, while in 3 testosterone treated controls the average increase was from 2,220,000 to 3,340,000, showing a slightly greater increment than the capons receiving double injections. Three control capons receiving α-estradiol alone showed an average decrease from 2,620,000 to 2,180,000. The results of the stilbestrol testosterone combination were varied, one showed an increase from 2,290,000 to 3,010,000, and 2 decreases from 2,830,000 to 2,540,000, and 3,230,000 to 2,790,000 after 25 injections. The average red cell count for these 3 capons showed a rise from 2,783,000 to 3,010,000 after 15 injections and a drop to 2,780,000 after 25 injections, which is lower at both points than the average obtained from testosterone injected capons. (See Table I).

Conclusions. From these results we conclude that one of the factors responsible for the higher erythrocyte number in male fowl is the presence of androgen while the lower number in the female is caused either by a lack of an adequate amount of androgen or by the presence of estrogen. The mechanism by which the increase is brought about is not known, but the decrease may be due to deposition of endosteal bone which would interfere with erythropoiesis.[5,6] The results obtained from combined androgen and estrogen injections are not conclusive, although there is some indication that the stimulating effect of androgen on red cell formation is inhibited in some way by estrogen.

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[4] Domm, L. V., and Davis, David E., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 665.

[5] Bloom, M. A., Bloom, W., Domm, L. V., and McLean, F. C., *Anat. Rec.*, 1940, **78**, 143.

[6] Bloom, W., and Domm, L. V., *Anat. Rec.*, 1941, **81**, 91.

14020 P

Nigrohypothalamic Fibers in the Cat.

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Marchi experimental studies (Papez,[1] Bodian,[2] Fox[3]) have shown that the mammillary peduncle is an ascending fasciculus. According to Ganser[4] the fibers constituting the mammillary peduncle arise from the *Substantia nigra.* Recently Papez[5] also has suggested that the *Substantia nigra* contributes to the formation of this peduncle. Others (see Raymon y Cajal[6]), although not denying the presence of the nigral component of the peduncle, have reported that fibers from the contralateral nucleus gracilis course forward with the medial lemniscus, and then pass through the nigra to enter the mammillary peduncle.

This report is based upon the results of experiments performed in an attempt to determine the efferent connections of the *Substantia nigra.* It is primarily concerned with fibers of nigral origin contained within the mammillary peduncle and the termination of such fibers in the hypothalamus.

Materials and Methods. Employing the Horsley-Clark apparatus, lesions were produced in the brain stems of 27 cats. Operations were performed under sodium barbiturate anesthesia using aseptic technic. All animals were sacrificed on the twelfth to fourteenth day postoperatively and the desired portions of the brains treated according to the Swank and Davenport[7] modification of the Marchi technic. Nitrocellulose was used for embedding and every third-40μ section was mounted for study. Nuclear relations were checked in a normal cat brain, sectioned transversely and stained with cresyl violet.

In 22 animals the lesions involved the *Substantia nigra;* in 3, the lesions were located in the mesencephalic tegmentum just dorsal to the nigra and in 2, multiple lesions were located in the basilar and the ventral tegmental areas of the pons.

Observations and Discussion. Fibers arising from the *Substantia nigra* course to the hypothalamus by way of the homolateral mammillary peduncle. Such fibers are degenerated in all experimental animals which have lesions in the *Substantia nigra,* and they vary in quantity directly with the degree of involvement of the nigral complex.

These fibers reach the mammillary peduncle by two routes. Some pass directly medialward to the peduncle through the nucleus interpeduncularis transversi and the nucleus of the mammillary peduncle. Others pass ventromedially to enter the medial part of the basis pedunculi within which they ascend for variable distances and then turn medially into the peduncle.

These two groups of fibers intermingle in the lateral part of the mammillary peduncle as they ascend to the hypothalamus. They are supplemented by additional fibers from the *Substantia nigra* at each successive rostral level throughout the length of the nigral complex. Thus, the nigral component accounts to a large extent for the increase in the size of the peduncle as it proceeds rostrally (Ganser[4]).

The degenerated nigral fibers are distributed within the hypothalamus to the following homolateral cell groups: 1, the lateral mammillary nucleus; 2, the medial mammillary nucleus; 3, the supramammillary nucleus; 4, the lateral hypothalamic nucleus (the lateral hypothalamic area); and 5, the ventrolateral hypothalamic nucleus. A large number of the degenerated fibers continue directly forward from the peduncle to terminate in the lateral mammillary nucleus. Others swing ventromedially through the lateral mammillary nucleus to terminate in the ventrolateral hypothalamic nucleus. Still other fibers course dorsomedially and terminate both in the rostral part of the medial mammillary nucleus and in the supramammillary nucleus. Laterally located degenerated fibers of the mammillary peduncle continue forward to terminate in the lateral hypothalamic nucleus. These fibers are distributed throughout the lateral hypothalamic area forward almost to the level of the optic chiasma.

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[1] Papez, J. W., *Anat. Rec.*, 1923, **25**, 146.

[2] Bodian, D., *J. Comp. Neur.*, 1940, **72**, 207.

[3] Fox, C. A., *J. Comp. Neur.*, 1941, **75**, 411.

[4] Ganser, S., *Morph. Jahrb.*, 1882, **7**, 591.

[5] Papez, J. W., *J. Comp. Neur.*, 1938, **69**, 103.

[6] Raymon y Cajal, S., *Histologie du système nerveux de l'homme et des vertébrés*, 1911, A. Maloine, Paris.

[7] Swank, R. L., and Davenport, H. A., *Stain. Tech.*, 1935, **10**, 87.

The available experimental material indicates that the *Substantia nigra* does not contribute to the crossed component of the mammillary peduncle. Lesions in the *Substantia nigra* produce no degeneration which terminates in the contralateral hypothalamus excepting when they are located in the caudomedial part of the nigra. Lesions in this part of the nigra interrupt fibers entering the mammillary peduncle from the ventral tegmental nucleus (Fox[3]). The crossed degeneration present in 4 of our animals can be attributed in each case to the interruption of these fibers. The distribution of the fibers of the mammillary peduncle presented here is in agreement with the termination of the peduncle as described by other investigators (see review of Huber and Crosby[8]).

Careful consideration has been given in the present study to determine the exact origin of degenerated fibers in the mammillary peduncle. Experimental evidence indicates that the peduncle arises in part from the mesencephalic tegmentum. Fox[3] has shown the ventral tegmental nucleus to be the specific nucleus of origin for these fibers. Tegmental lesions located lateral and rostral to this nucleus produce no degeneration in the mammillary peduncle. Likewise no degeneration occurs in the mammillary peduncles of animals with multiple lesions in the pons, caudal to the nigra. Thus the degeneration produced within the mammillary peduncle in animals with nigral lesions apparently results from injury to the nigral cells.

Summary. Degenerated fibers, resulting from lesions located in the *Substantia nigra*, ascend to the hypothalamus by way of the homolateral mammillary peduncle. These fibers terminate in homolateral nuclei of (a) the mammillary bodies, (b) the infundibular region and (c) the lateral hypothalamic area. None of the degenerated nigral fibers appear to enter the contralateral hypothalamus.

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[8] Huber, G. C., and Crosby, E. C., *Arch. Neur. and Psychiat.*, 1929, **22**, 187.

14021

Influence of Respiratory Inspiration on the Heart Rate.

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It is well known that deep sustained inspiration is associated with a slowing of the heart rate. Of the recognized mechanisms having this effect the following may be mentioned: (1) overdistention of the lungs, evoking the cardioinhibitor reflex described by Anrep, Pascual and Rössler[1] and by von Saalfeld;[2] (2) the "Lungenentlastungsreflexe" of Schwiegk,[3] in which an increase of pressure in the pulmonary vessels slows the heart; and (3) the carotid sinus and aortic cardioinhibitor reflexes activated by a rising systemic arterial pressure. Certain effects of inspiration would tend to accelerate the heart: (1) increased venous inflow into the thorax evoking the Bainbridge reflex; (2) moderate distention of the lungs, stimulating the accelerator reflex of Anrep *et al.*[1] and von Saalfeld;[2] (3) inhibitory irradiation from the respiratory center to the cardiac vagus center;[1] and (4) the voluntary effort exerted in the act of inspiration. In an attempt to evaluate these factors in human subjects, the effects of chest position and of reduction of intrapulmonary pressure in various chest positions was studied.

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[1] Anrep, G. V., Pascual, W., and Rössler, R., *Proc. Roy. Soc.*, 1936, **119B**, 191, 218.

[2] von Saalfeld, E., *Pflügers Arch.*, 1932, **231**, 724.

[3] Schwiegk, H., *Pflügers Arch.*, 1935, **236**, 206.

The intrapulmonary pressure, assumed to be atmospheric when the chest was held in any given position with the glottis open, was reduced by the following procedure: the nose was clamped and a mouthpiece connected with a mercury manometer was held in the mouth; the subject, lying in the supine position, watched the manometer and made a voluntary inspiratory effort sufficient to reduce and maintain the pressure at –20 mm Hg for at least 30 seconds. Observations were made with the chest held voluntarily in the position of full inspiration, with and without reduction of intrapulmonary pressure, and in the position of full expiration with and without reduction in intrapulmonary pressure. Heart rate was recorded continuously on a kymograph by means of a Gaertner sphygmograph. Arterial pressures were determined by the auscultatory method. Before each experiment the subject rested for 15 minutes, at the end of which control heart rate and arterial pressure determinations were made. Between trials, sufficient time was allowed to elapse for the normal levels to return. Eleven experiments were performed on 6 subjects, 4 males and 2 females.

The average values for heart rate, and systolic and diastolic pressures, at various times after the assumption of the appropriate chest position (with and without reduction of intrapulmonary pressure), are shown in the accompanying figures. The following discussion will be confined to consideration of the heart rate 20 seconds after the beginning of the trial, since the arterial pressures at this time were not yet affected by the respiratory arrest.

The assumption of the chest position of full inspiration is followed by a reduction of the heart rate averaging 11 beats per minute at 20 seconds. This fall is statistically significant, the probability of chance occurrence being less than 1 in 1000. None of the three other maneuvers produced statistically significant changes in heart rate.

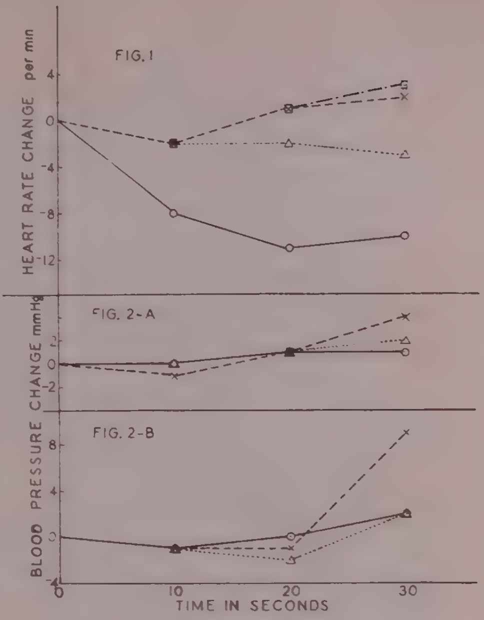

FIG. 1.

Changes in heart rate and blood pressure accompanying changes in chest position and intrapulmonary pressure: Fig. 1, heart rate; Fig. 2-A, systolic pressure; Fig. 2-B, diastolic pressure. Continuous line, inspiration at atmospheric pressure; Dotted line, inspiration at –20 mm Hg.; broken line, expiration at atmospheric pressure; alternate dots and dashes, expiration at –20 mm Hg.

Are any of the known cardioinhibitor reflexes which might be evoked by inspiration adequate to explain the concomitant slowing of the heart rate? Since the arterial pressure is not altered, the carotid sinus and aortic reflexes cannot be responsible. It is improbable that the degree of pulmonary distention which could be brought about under these conditions would be adequate to evoke the reflex slowing of the heart, for it requires, according to Anrep and his associates,[1] inflation with a positive pressure which in an open chest would cause the lungs to bulge out of the thorax. There remains only the reflex of Schwiegk,[3] which might be activated by a rise in pressure in the pulmonary vessels produced by increased output of the right ventricle resulting from the greater venous inflow into the chest in inspiration. In order to be effective in slowing the heart, the Schwiegk reflex must outweigh the accelerator influence of the Bainbridge reflex which would also be elicited under these conditions.

If inspiration slows the heart by the consequences of reduction in intrathoracic pressure, the slowing should appear when the intrathoracic pressure is reduced by other means. Voluntary inspiration against resistance is one such means and the one which we have employed. However, when the intrapulmonary pressure is reduced to –20 mm Hg with the chest in the expiratory position, there is no slowing of the heart. Probably the Bainbridge reflex and the Schwiegk reflex, both of which are doubtless evoked, cancel each other's effect on the heart rate.

Thus we arrive at the conclusion that none of the cardioinhibitor reflexes discussed can explain the slowing under consideration. Some other factor must be postulated, such as the position of the chest itself or of the diaphragm as suggested by Sylla,[4] or some secondary consequence of the inspiratory position other than those already discussed.

In support of this conclusion may be cited the finding that the heart rate when the chest is in the inspiratory position with an intrapulmonary pressure of –20 mm Hg is somewhat slower than when the chest is in the expiratory position with the same intrapulmonary pressure. The fact that the slowing is not as great as when the intrapulmonary pressure is atmospheric may be attributed to the greater voluntary effort required to reduce the intrapulmonary pressure to –20 mm Hg when the chest is in the inspiratory position than when it is in the expiratory position. The cardiac acceleration accompanying the greater voluntary effort, it may be suggested, would increase the inhibitory irradiation from the respiratory center to the cardiac vagus center and so in part counteract the decelerating influence of the inspiratory chest position.

Summary and Conclusion. The slowing of the heart produced by sustained deep inspiration is not due to the reflex consequences of lowered intrathoracic pressure nor to any other hitherto recognized reflex mechanism. It is probably caused by some influence activated by the assumption of the inspiratory position of the chest.

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[4] Sylla, A., *Klin. Woch.*, 1936, **15**, 849.

14022 P

Concentrations of Sulfadiazine in Human Saliva Attained by Chewing Paraffin Containing Sulfadiazine.

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It is now recognized that the sulfonamides are effective in the therapy of many severe infections of hemolytic streptococcic origin. The results with these compounds have been less satisfactory in the treatment of patients with streptococcic upper respiratory diseases. This is especially true of the milder types of infections such as acute pharyngitis and tonsillitis. In our own clinical experience, the ingestion of moderate doses of the sulfonamides by patients having acute pharyngitis and tonsillitis has occasionally been attended by disagreeable side effects from the drugs, which overshadowed the questionable therapeutic responses. We have attempted to treat such individuals by the local application to the pharynx of powdered sulfonamide preparations, or solutions and suspensions of the compounds. The results have been disappointing.

The possibility arose that more desirable therapeutic responses might be obtained if the tonsillar tissues and oral pharynx were subjected to high concentrations of a suitable

TABLE I.
Sulfadiazine Concentrations in Saliva and Blood of Individuals Chewing 1.5 g Paraffin Impregnated with 0.325 g Sulfadiazine.

Patient No.	Mg per 100 cc sulfadiazine in saliva 5 min	30 min	1 hr	2 hr	Mg per 100 cc sulfadiazine in blood 1 hr
1	338	307	308	400+	1.48
2	70.8	310	278.6	112.2	0.59
3	44.3	115.9	268	342	0.67
4	45.2	888	888	359	0.30
5	35.4	109	309	334	0.00
6	106	295	325	65	trace
7	72.2	354	56	32.4	0.88
8	366	590	228	91.3	1.18
9	20.6	103	285	320.0	0.88
10	32.4	400	216	29.5	trace
11	65	516	272	79.5	0.88
12	46.8	173	120	41	0.80

sulfonamide over a considerable period of time. Clinical and *in vitro* studies indicate that sulfadiazine possesses a high degree of antibacterial activity for Lancefield group A strains of hemolytic streptococci, which may cause acute tonsillitis, acute pharyngitis, as well as scarlet fever. An approach to this problem was evolved by mixing sulfadiazine with melted paraffin, and having human subjects chew on the hardened paraffin. In this manner, remarkably high concentrations of sulfadiazine were maintained in the saliva. At this time, we are presenting a report of these observations.

Paraffin blocks, suitable in size for chewing, were impregnated with crystalline sulfadiazine in the following manner: 4.2 g (60 grains) of sulfadiazine was thoroughly mixed with 15 g of melted paraffin. The paraffin was then poured into a mold such as used for making twelve standard-sized rectal suppositories. Under these conditions, each "suppository" contained 0.375 g sulfadiazine, or slightly more than 5 grains. Human subjects were instructed to chew the paraffin, and samples of saliva were collected at stated intervals for the determination of sulfadiazine (unconjugated) concentrations. The determinations were made according to the method of Bratton and Marshall.[1]

The results with one group of 12 subjects are presented in Table I. Samples of saliva for sulfadiazine determinations were collected at the end of 5 minutes and 30 minutes, and after one and 2 hours. A specimen of venous blood was obtained after the paraffin had been chewed for one hour.

A second group of 8 patients was given a gelatin capsule containing 0.325 g of sulfadiazine. Samples of saliva were collected at the same intervals as in the preceding group, and samples of venous blood were obtained after the paraffin had been chewed for one, 2, and 3 hours. The sulfadiazine concentrations are shown in Table II.

TABLE II.
Sulfadiazine Concentrations in Blood of Individuals Receiving by Mouth One Gelatin Capsule Containing 0.325 g of Sulfadiazine.
Under same treatment there was no sulfadiazine in 100 cc saliva when tested 5, 30 min, 1, 2 hr.

Patient No.	Mg per 100 cc sulfadiazine in blood 1 hr	3 hr	6 hr
13	1.03	2.06	2.06
14	1.04	1.40	1.92
15	0.60	0.80	1.03
16	0.60	0.90	2.20
17	1.20	1.48	1.48
18	trace	1.48	2.36
19	1.48	2.06	1.62
20	1.20	1.48	2.10

It is of interest that in individuals receiving full therapeutic doses of sulfadiazine so that blood concentrations of sulfadiazine were maintained between 5 and 10 mg per 100 cc, the levels of sulfadiazine in the saliva ranged between 3 and 4 mg per 100 cc.

A study of the therapeutic results with the paraffin-sulfadiazine preparation in patients with acute pharyngitis or tonsillitis of streptococcic origin has not progressed far enough to warrant any conclusions at this time. This investigation will include cultures of material from the oral pharynx before, during, and after treatment with paraffin-sulfadiazine. The observations made to date are given so that other groups may carry out investigations relative to the therapeutic value.

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[1] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

Summary. When 0.325 g of sulfadiazine is incorporated into approximately 1.5 g of paraffin, and the paraffin-sulfadiazine mixture is chewed by human subjects, high concentrations of free sulfadiazine were attained in the saliva. Such a procedure may favorably affect the course of acute hemolytic streptococcic infections such as pharyngitis and tonsillitis without recourse to larger systemic doses.

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Immunologic Studies in Experimental *Trypanosoma cruzi* Infections: 2. Slide Agglutination and Intradermal Tests.*

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Whereas the antigenic composition of bacteria is fairly well known, little work has as yet been done on the antigenic composition of protozoa pathogenic to man. Flagellar 'H' and somatic 'O' agglutinogens have been demonstrated by the writer[1] for *Leishmania tropica,* and positive skin tests have been obtained by the writer[1] with the polysaccharide and protein fractions of *L. tropica* in cutaneous leishmaniasis.

The Machado[2] complement fixation test, using as antigen glycerine and aqueous extracts of the heart and spleen of infected puppies, has been used for the diagnosis of "Chagas" disease in man, and more recently[3] cultures of *T. cruzi* have been utilized as antigen. Precipitin[4] and agglutination[5] tests have also been used in experimental *T. cruzi* infections. Rabbits were immunized with killed and living cultures of *T. cruzi,* and their agglutinin titers ranged from 2,000 to 130,000, with an average of 2,000 to 4,000. Intravenous method of immunization gave rise to higher titers. Rabbits and guinea pigs which were given living cultures showed a lower titer (256 to 1,024). Sera of animals infected with *T. brucei* or *T. hippicum* agglutinated *T. cruzi* in very low titers (1:32). Lysins[6] for *T. cruzi* have also been observed.

Preparation of antigens for immunization. Five strains of *T. cruzi,* 2 arthropod strains from Texas (Packchanian, Davis), one armadillo strain from Brazil (Tatu) and 2 human strains, one from Brazil (1114) and another from Panama, were each cultured on modified Senekjie's medium[7,8] in Roux bottles or Erlenmeyer flasks for 7 to 10 days. The growths were emulsified in physiologic salt solution and killed in 0.3 % formalin. After 24 hours the trypanosomes were collected by centrifugation, and were suspended in 0.5 % phenol in saline. The suspension was standardized to contain 30 million trypanosomes per cc (by making counts in the blood-counting chamber).

Rabbits were immunized with respective cultures by intravenous injections. The dosage

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[1] Senekjie, H. A., *Am. J. Hyg.*, 1941, **34**, Sec. C. 63.

[2] Guerreiro, C., and Machado, A., *Brasil Medico,* 1913, **23**, 225.

[3] Kelser, R. A., *Am. J. Trop. Med.*, 1936, **16**, 405.

[4] Packchanian, A., *J. Immunol.*, 1935, **29**, 84.

[5] Packchanian, A., *Publ. Health Rep.*, 1940, **55**, 2116.

[6] Denison, N., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 26.

[7] Senekjie, H. A., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1939, **33**, 267.

[8] Senekjie, H. A., unpublished data.

was gradually increased from 0.5 cc until 1 cc doses were reached. Twelve such injections were given twice a week. Ten days after the last injection, a trial bleeding was carried out by cutting the ear vein, and the titer of agglutinins was determined. Two weeks after the last injection of killed trypanosomes, immunization was started with living trypanosomes. The injections were given intravenously once a week for a period of 2 months. Skin tests were performed by injecting these animals intradermally with 0.2 cc of living and dead cultures of trypanosomes. The rabbits were then bled and the homologous agglutinin and lysin titers determined. At no time could living trypanosomes be found in the peripheral blood. Two of the rabbits died and sections of the various organs did not reveal any leishmania stages of the trypanosome.

Preparation of antigens for tube agglutination. 1. *Flagellar 'H' antigen.* The trypanosomes were grown on Senekjie's medium[7,8] at room temperature for 10 days. The colonies were emulsified in physiological salt solution containing 0.3% formalin, and the suspension left at room temperature for 24 hours. The trypanosomes were then collected by centrifugation, resuspended in saline and kept in the ice chest. The suspensions were standardized to contain 30 million trypanosomes

2. *Somatic 'O' antigens.* The trypanosomes were grown as before. The colonies were emulsified in saline, the trypanosomes collected by centrifugation, and resuspended in 90 % alcohol, kept at room temperature for 24 hours, then washed three times in saline, suspended in saline to contain 30 million trypanosomes per cc and stored in the ice chest.

Preparation of antigen for slide agglutination. The Tatu (*i.e.*, armadillo) strain was very smooth, and formed a very homogeneous suspension. The antigen was prepared from the Tatu strain. It was grown on Senekjie's medium at room temperature for 10 days. The colonies were emulsified in physiological salt solution containing 0.5 % phenol, and standardized to contain 100 million trypanosomes per cc. When kept in the ice box, the trypanosomes remained alive for about a week.

Tube agglutination test. The sera of immunized rabbits were diluted in series in physiological salt solution and equal amounts of homologous 'H' and 'O' antigens were added. The tubes were incubated at 37°C overnight and 2 readings were taken, one immediately after and the second 5 hours after the racks were placed at room temperature. No difference was noted between the two readings. Tube agglutination was performed using the antigen prepared for the slide agglutination but diluted to contain 30 million of organisms per cc.

Slide agglutination test. One and one-half-inch squares were marked off on a glass plate. To various dilutions of each serum in the different squares an equal amount of homologous antigen was added. The mixtures were stirred with a match stick and the plate was gently shaken so that there was a uniform mixing of the serum and antigen. Agglutination became apparent in one to two minutes and was complete in 3 to 5 minutes. The agglutination was very marked in the presence of low dilutions of sera but became progressively less and less marked as the dilution was increased until the end titer was reached. Negative controls were included in these tests.

Lysis. Living trypanosomes were suspended in various dilutions of the immune sera and kept at room temperature, and at intervals were examined. Controls were included, using normal serum and physiological salt solution.

Results. Table I shows the agglutinin titers of the sera of rabbits immunized with killed cultures. The 'O' titers were lower than the 'H' titers. Those for the slide agglutination were roughly half those for the 'O.'

Table II shows the titers of the sera of the rabbits which were given living cultures of trypanosomes following the killed trypano-

TABLE I.

Agglutination Titers of Rabbits Following Immunization with Killed Cultures of *T. cruzi.*

Serum	Tube agglutination Homologous 'H' antigen	Homologous 'O' antigen	Slide agglutination
Packchanian	1:1,600	1:400	1:100
Davis	1:400	1:200	1:100
Tatu	1:400	1:200	1:100
1114	1:400	1:200	1:50
Panamá	1:400	1:200	1:200

TABLE II.

Agglutination Titers of Rabbits Following Immunization with Killed and Living Cultures of *T. cruzi*.

Serum	Tube agglutination		Slide agglutination
	Homologous 'H' antigen	Homologous 'O' antigen	
Packchanian	1:3,200	1:1,600	1:1,600
Davis	1:1,600	1:800	1:800
Tatu	1:6,400	1:1,600	1:1,600
1114	1:3,200	1:800	1:400
Panamá	1:6,400	1:800	1:800

somes. These titers were higher and ranged from 1:1,600 to 1:6,400 in the case of the 'H' agglutinins, and 1:800 to 1:1,600 in the case of 'O' agglutinins. There was more correlation between the slide agglutination and the 'O' agglutination.

Table III shows the relation between the slide agglutination and the tube agglutination, using the Tatu antigen for the tube and slide agglutination. The titers for the tube agglutination ranged from 1:1,600 to 1:6,400, while the slide agglutination ranged from 1:800 to 1:1,600 with a ratio of about 2:1 to 4:1.

TABLE III.

Comparison of the Tube and Slide Agglutination Titers Using Séra of Rabbits Immunized with *T. cruzi* (Tatu antigen used)

Serum	Tube agglutination	Slide agglutination
Packchanian	1:1,600	1:400
Davis	1:1,600	1:800
Tatu	1:6,400	1:1,600
1114	1:6,400	1:1,600
Panamá	1:6,400	1:1,600

The flagellar 'H' agglutination was in large flakes, and settled to the bottom of the tube, leaving a clear supernate. On shaking the tube the large flakes were broken up and a fine homogeneous suspension with very fine clumps was produced. The somatic 'O' agglutination was in small flakes, and there was a general turbidity of the fluid in the tube due to small clumps. Some of the clumps settled to the bottom of the tube but never broke up into finer clumps on shaking the tube. In the case of the slide tests the agglutination clumps were rather large and resembled those of the large flaking type. They appeared first on the margin of the drop and progressively the whole drop showed the agglutination.

Skin tests. Following the intradermal injection of the cultures of *T. cruzi* into the skin of immunized rabbits no reaction was noticed for a period of 12 hours, but later a red papule was formed which increased in size to a 5-cent piece and reached its height in 24 hours, fading away in 3 days.

Lysis. The immune sera lysed the trypanosomes in 1:100 dilution in 2 hours at room temperature. Saline and normal serum controls showed no change. At first the motility ceased and progressively the trypanosomes disintegrated.

A guinea pig which was inoculated with trypanosomes recovered from the rectum of an assassin bug developed infection, and its blood showed an agglutinin titer of 1:400. A second guinea pig infected with *T. equiperdum* agglutinated *T. cruzi* in 1:20 dilution.

A hundred Wassermann sera were tested by the slide agglutination method, and all were found to be negative. Two acute tertian malaria cases and 5 paretics who had induced quartan malaria also gave negative slide agglutination. One patient suffering from an undiagnosed fever gave a titer of 1:10.

Discussion. The same types of antibody are present in *Trypanosoma cruzi* infections as are present in bacterial infections, *i.e.*, complement fixing antibodies,[2,3] precipitins,[4] agglutinins[5] and lysins.[6] The writer confirms the findings of Packchanian but shows that the agglutinin consists of 'H' and 'O' types. He also confirms the findings of Denison on the presence of lysins, and provides evidence on the presence of a skin-sensitizing antibody which gives rise to positive skin tests.

In bacterial infections it is claimed that the 'O' agglutinin is the one which protects against infections. The importance of 'H' and 'O' agglutinins is not yet known in *Trypanosoma cruzi* infections. It is very probable that the 'H' antigen is located somewhere in the flagellum or in the undulating membrane, and the 'O' antigen in the body of the trypanosome. These two antigens may be of great importance in the diagnosis and the protective vaccine in Chagas' disease in man.

The slide agglutination test is very easy to perform and requires very little equipment. It is of particular value when large numbers of sera are to be examined to find suspected cases. Xenodiagnosis needs laboratory-bred assassin bugs, making it a complicated test to perform. The same thing holds true of biopsy material. A simple, easy serological test would be of great value in diagnosis of Chagas' infection in man, such as the slide agglutination test.

Summary and conclusions. 'H' and 'O' agglutinins, skin sensitizing antibodies and lysins are present in the blood of animals infected or immunized with *Trypanosoma cruzi.* A simple, easy slide agglutination test is described for the diagnosis of experimental *Trypanosoma cruzi* infection in animals. Out of 108 human sera obtained from a hospital laboratory, only one agglutinated *T. cruzi* in 1:10 dilution.

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Effect of Hematin Upon Stability of Thiochrome in Solutions of Alkaline Ferricyanide.*

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The usual technic for determining thiamin by the thiochrome reaction consists in converting thiamin to thiochrome by alkaline oxidation with ferricyanide, extracting the thiochrome with iso-butyl alcohol and measuring its fluorescence with a photo-electric fluorometer.[1] In the course of a study of the thiamin inactivating enzyme of carp,[2] it has been observed that this procedure of analysis gives erratic results in the presence of hemolyzed red blood cells. Closer investigation has indicated that traces of hematin markedly increase the rate of destruction of thiochrome in solutions of alkaline ferricyande. In the presence of this pigment before the thiochrome formed by alkaline oxidation of thiamin can be extracted with iso-butyl alcohol, a considerable portion may be destroyed and falsely low values of thiamin analysis obtained.

TABLE I.

Effect of Hematin upon the Rate of Destruction of Thiochrome in Solutions of Alkaline Ferricyanide.

	Time in minutes						
Solution	0	1	3	5	10	20	30
Control	47.0*	47.0	46.5	46.5	45	44.5	43.0
Hematin (1:1,000,000)	46.0	6.0	1.5	1.5	0.5	0	0
Hematin (1:10,000,000)	46.5	37.5	28.5	22.0	10.0	3.0	2.0

*Fluorescence in galvanometer units, Coleman Electronic Photofluorometer—Model 12.

Table I illustrates the disappearance of thiochrome fluorescence which follows addition of 1 ml of a dilute solution of hematin[†] to 9 ml of an aqueous solution of 0.82 μg of thiochrome containing alkali and ferricyanide in amounts comparable to those used in the thiochrome technic of thiamin estimation. The hematin effect is dependent upon the presence of both alkali and ferricyanide in the solution and may be observed with high dilutions of hemolyzed red blood cells as well as with dilutions of hematin.

Boiling, peptic digestion, carbon monoxide, or preliminary treatment with strong acid or alkali does not diminish the activity of the

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[1] Jansen, B. C. P., *Rec. d. Trav. Chim. d. Pays-Bas.*, 1936, **55**, 1046.

[2] Owen, Philip S., and Ferrebee, Joseph W., in press.

[†] Crystalline preparation of chlor-hemin obtained from Dr. Otto Schales, Peter Bent Brigham Hospital, Boston, Massachusetts.

hematin solution. However, if significant protein precipitation occurs in the presence of the hematin compound, the active material is adsorbed on the protein and taken out of solution. Under these circumstances the precipitated material becomes active and the supernatant fluid becomes inactive.

The mechanism of the hematin effect has not been studied in detail. Preliminary observations have failed to indicate a combination of thiochrome and hematin of the parahematin type. The most likely explanation is that hematin catalyzes the oxidative destruction of thiochrome in a manner similar to that described by Harrison in the case of cysteine.[3]

Summary. Hematin markedly accelerates the destruction of thiochrome in solutions containing alkaline ferricyanide.

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[3] Harrison, D. C., *Bioch. J.*, 1924, **18**, 1009.